REMARKS

Reconsideration of the application is respectfully requested.

Claims 120-131, 144-193, and 205-224, directed to non-elected subject matter, and claims 141-143 have been cancelled. Claims 132-140 and 194-204 are pending and under examination. Claims 132, 197 and 198 have been amended. No new matter was added.

Restriction Requirement

Applicants affirm their election, without traverse, of Invention Group II, claims 132-143 and 194-204.

Rejection under 35 USC Section 112

Claims 141-143 and 202-204 stand rejected as containing subject matter not present in the specifications as originally filed.

Claims 141-143 are cancelled and the rejection is therefore moot.

As to claims 202-204, Applicants respectfully traverse. For example, page 3, line 2, as amended pursuant to §§ 608.01(p) and 2163.07(b), discloses a combination of cocoa procyanidins and a cyclo-oxygenase (COX) inhibitor, and page 15, lines 5-7 discloses aspirin as a COX inhibitor. The amended text is from the parent '661 application, which is incorporated by reference in the present application. Accordingly, withdrawal of the rejection is respectfully requested.

Claims 197-198 are rejected as being indefinite for being improperly dependent on claim 194. The claims have been amended to recite a method rather than a composition. Applicants apologize to the Examiner for this inadvertent error. Withdrawal of the rejection is respectfully requested.

Rejection under 35 USC Section 103

Claims 132-140 and 197-198 stand rejected as obvious over Clapperton *et al.* The Examiner states that Clapperton discloses compositions comprising polyphenols (procyanidins) from cocoa, which compositions are made into liquor. The Examiner concludes that it would have been obvious to a person of skill in the art to make additional procyanidin containing

compositions by packaging the compositions disclosed by Clapperton with instructions on methods of use. Applicants respectfully traverse the rejection.

Claims 132-140, as now amended, recite articles of manufacture comprising a composition comprising a cocoa procyanidin monomer and/or oligomer *in the amount effective to inhibit platelet aggregation*. Additionally, the composition comprises a carrier, which is a pharmaceutical, veterinary, dietary supplement or food carrier, *i.e.*, a *carrier designed for therapeutic delivery* of the cocoa procyanidin monomer and/or oligomer to a subject in need thereof. Finally, the article of manufacture comprises instructions for use, which functionally define (and thus limit) the composition as a *therapeutic* composition, *i.e.,* the composition that, when administered, has a therapeutic effect. In other words, the recitation of the instruction for use functionally defines the amount of cocoa procyanidin monomer and/or oligomer as the amount effective for the treatment recited in the claims. Clapperton fails to teach or suggest such articles of manufacture for the reasons shown below.

Clapperton fails to suggest any article of manufacture comprised of a therapeutic composition containing an effective amount to inhibit platelet aggregation and instructions for therapeutic use. Clapperton teaches that cocoa beans can be dehusked and milled into liquor and that defatted cocoa powder may be used to make extracts. He also states that he isolated various procyanidin fractions. Clapperton does not teach or suggest, however, that cocoa procyanidin monomers and/or oligomers have any therapeutic effects and, therefore, cannot suggest manufacturing delivery vehicles with cocoa procyanidin monomers and/or oligomers for administration to humans or animals, nor can he suggest the therapeutically effective amounts that such compositions must contain.

Without suggestion of any therapeutic effects of these compounds by Clapperton, a person of skill in the art would *not* have been motivated, with any reasonable expectation of success, to combine the compounds in the anti-platelet effective amounts with carriers adapted for therapeutic applications and prepare articles of manufacture claimed herein. In fact, in view of Clapperton's teaching that the compounds have unpleasant bitter and astringent taste, a person of skill in the art would have avoided adding the compounds into any composition, e.g. food, intended for human or animal consumption without a suggestion of the compounds' therapeutic benefits.

In view of the above, the invention of claims 132-140, *as a whole,* would *not* have been obvious to a person of skill in the art as of the effective filing date. Applicants, therefore, believe that the withdrawal of the Clapperton rejection is in order. Such action is respectfully requested.

As to claims 197-198, they are amended to recite methods, as it was originally intended. Through an inadvertent error, the claims recited compositions. Thus, the rejection over Clapperton is now believed to be moot.

* * *

Method claims 194-196 and 199-201 stand rejected as obvious over Chang in view of Clapperton. The Examiner states that Chang teaches anti-platelet therapy using procyanidin oligomers, and Clapperton teaches that cocoa contains procyanidins. Applicants respectfully traverse the rejection.

The procyanidin monomers and/or oligomers recited in claims 194-196 and 199-201 act on several mechanisms (discussed below) in the body of a human or a veterinary animal resulting in a comprehensive anti-platelet effect. Such unexpected efficacy of the claimed methods of anti-platelet therapy was not suggested by, or predictable from, the teachings of Chang and Clapperton.

Platelet activation and aggregation is a result of a complex series of events involving multiple membrane receptors, second-messengers and signaling pathways, requiring cellular interactions between platelets and endothelial cells as well as between platelets themselves, and characterized by platelet shape change and secretion of granules. The intra-platelet free calcium levels are also critical for the activation status of the platelet. The complexity of these mechanisms is illustrated in Figure 1 of Blockmans et al., Platelet Activation, *Blood Review* (1996), 9:143-156 (Attachment I).

Applicants have discovered unexpected effects of the procyanidin monomers and/or oligomers recited in the present method claims on several mechanisms and molecules involved in platelet activation and aggregation:

First, the compounds recited in the Applicants' method claims *increase nitric oxide (NO) synthesis* (*see e.g.* specification, pages15-16). NO plays an important role in platelet signaling and function. As illustrated in Attachment II, Figure 1, page 758, of Loscalzo, Nitric Oxide Insufficiency, Platelet Activation, and Arterial Thrombosis, *Circulation Res.*, (2001), 88:756-762, NO derived from endothelial cells or from platelets suppresses platelet activation by

activating guanylyl cyclase (GC), enhancing Ca-dependent refilling of calcium stores, and inhibiting activation of PI3K. The former two mediate second-order effects resulting in suppression of calcium influx and leading to suppression of P-selectin expression and of expression of the active conformation of GPIIb/IIIa receptor. This results in inhibition of platelet aggregation.

The anti-platelet aggregation effects mediated by NO were entirely unexpected. In fact, as of the effective filing date of the present application, persons of skill in the art believed that flavonoids were NO scavengers, *i.e.,* that they reduced the available level of NO, which would have resulted in the entirely opposite effects of the ones described above. (*see* Attachment III, van Acker *et al.,* Flavonoids as Scavengers of Nitric Oxide Radicals, *Biochem. Biophys. Res. Commn.*, 214: 755-759 (1995)). There is no teaching or suggestion in Chang and Clapperton, individually or in combination, that the compounds recited in claims 194-196 and 199-201 can prevent platelet aggregation *via* NO.

Second, the compounds recited in the Applicants' method claims *increase prostacyclin (PGI_2) release* by endothelial cells (*see e.g.* specification, Example 13, pages 47-50, Figure 19A). As illustrated in Attachment IV, Figure 2, page 189, of Venkataraman et al., Platelets and Antiplatelet Drugs, *Indian J. Pharmacol.* (1992), 24:188-193, PGI_2 binds to a platelet membrane receptor which results in the elevation of platelet cyclic AMP (cAMP) concentration, which leads to inactivation of platelet myosin kinase, which in turn reduces actin-myosin interaction, platelet contraction and granule secretion (*see also,* Attachment IV, page 191, subsection (c)). There is no teaching or suggestion in Chang and Clapperton, individually or in combination, that the compounds recited in claims 194-196 and 199-201 can inhibit platelet aggregation *via* PGI_2. In fact, Chang experiments with platelets in isolation without taking into consideration the critical role of endothelial cells in platelet aggregation and thrombus formation.

Third, the compounds recited in the Applicants' method claims *decrease prostaglandin (PGE_2) release* by endothelial cells (*see e.g.* specification, Example 13, pages 47-50, Figure 19B). Reduction of prostaglandin is beneficial since it appears to be involved in platelet aggregation by priming protein kinase C and inhibiting cAMP formation (*see* Attachment I, page 149, 2nd col., sentence immediately above the title). There is no teaching or suggestion in Chang and Clapperton, individually or in combination, that the compounds recited in claims 194-196 and 199-201 can inhibit platelet aggregation *via* the decrease of PGE_2 production.

Finally, the compounds recited in the Applicants' method claims inhibit platelet activation and aggregation upon administration to a human—activation of the GPIIbIIIa receptor for adhesive proteins is inhibited, the expression of P-selectin is reduced, and platelet granule formation is decreased (*see* specification, Example 14, pages 50-56 and Figures 21 and 22). Receptor GPIIbIIIa and P-selectin, which are present on the surface of activated platelets, and platelet granules, which are secreted into the plasma, are the direct arbiters of platelet aggregation. They can be kept under control successfully using the method claims of the present invention. Chang and Clapperton fail to teach or suggest these direct anti-platelet effects.

As noted above, Chang has conducted experiments targeted to study an effect of several compounds on thromboxane (TXB_2) synthesis in the arachidonic acid pathway. However, while Applicants observed anti-platelet effects on several mechanisms and molecules, they did *not* observe an effect on TXB_2 (*see* specification, Figure 19C).

Aspirin is an example of a drug currently on the market, which acts by permanently inhibiting platelet TXB_2. Aspirin, however, has a significant draw back as it causes stomach bleedings in some patients. In view of the properties discussed above, Applicants consider their compounds, and methods of anti-platelet therapy recited herein, as an alternative to aspirin but without its side effects. This is yet another unexpected benefit of the present invention not suggested by the teachings of Chang in view of Clapperton.

Applicants therefore believe that the withdrawal of the prior art rejection of method claims is in order. An action to that effect is respectfully requested.

Information Disclosure Statement filed on February 25, 2002

Applicants respectfully request that the Examiner considers the Information Disclosure Statement (IDS) filed on February 25, 2002. The Examiner has returned the form PTO-1449 with the Official Action mailed March 21, 2002, but has not indicated that the references were considered. In fact, the Examiner has crossed out all the references cited by the Applicants as "Duplicative," presumably in view of the references cited in the parent application U.S. Ser. No. 08/831,245.

Applicants thank the Examiner for considering the references filed in the parent '245 application with respect to presently pending claims, and apologize for any duplicate entries made on the February 25, 2002 IDS.

However, it appears to Applicants that, with the exception of several references, the publications listed were not duplicative. Applicants, therefore, respectfully request that the Examiner considers these publications and indicate that they were considered on the attached clean copy of the PTO-1449 originally filed on February 25, 2002 (On the PTO-1449, Applicants have crossed out the publications that they determined were considered by the Examiner as a result of the earlier filed IDSs.)

If the binder of references submitted with the February 25, 2002, IDS is no longer available to the Examiner, it is respectfully requested that The Examiner telephone the undersigned and a new set of copies will be provided.

CONCLUSION

In view of the above amendments and remarks, Applicants believe that the application is now in condition for allowance. A notice to that effect is respectfully requested.

Respectfully submitted,

Date: February 14, 2003

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ATTACHMENT I

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BLOOD REVIEWS

Churchill Livingstone

BLOOD REVIEWS

Platelets

Platelet Activation

D. Blockmans, H. Deckmyn, J. Vermylen

SUMMARY. **This review article describes the different receptors, second-messengers and mechanisms involved in platelet activation. Several platelet agonists have well-defined receptors at the platelet membrane of which a number are single polypeptides with 7 hydrophobic transmembrane domains. These receptors are connected, via GTP regulatory proteins, with cytoplasmic second-messenger-generating enzymes. Phospholipase C and adenylate cyclase are the two best-known enzymes, generating inositol triphosphate (IP_3) and diacyl glycerol from phosphatidylinositol biphosphate and cyclic AMP from ATP respectively. The intraplatelet free calcium level, which is critical for the activation status of the platelet, is increased by IP_3 and is lowered in the presence of rising cyclic AMP concentrations. Shape-change occurs with small increases in intraplatelet calcium, while aggregation and secretion of granules take place at higher calcium levels. The role of myosin and actin filaments and of transmembrane glycoproteins is further discussed.**

The Role of Platelets in Health and Disease

Platelets play an essential role in the first phases of the haemostatic process. Whenever a blood vessel is damaged at its luminal side, subendothelial elements come into contact with the blood elements. Blood platelets become activated by subendothelial collagen, microfibrils and von Willebrand factor (vWF). This activation process makes platelets adhere to the exposed subendothelial tissues and to each other. This so-called 'white thrombus' closes up the gap in the injured blood vessel. Subsequent to platelet activation, the coagulation cascade will form fibrin fibrils, which strengthen the primary thrombus and transform it into a 'red thrombus'. For larger lesions, this coagulation process is indispensable for haemostasis. When there are too few platelets or if platelets are afunctional, primary haemostasis will be hampered what may result in a haemorrhagic diathesis.

On the other hand, an excessive platelet activation is held responsible for a variety of thrombo-embolic phenomena, especially on the arterial side. Platelet aggregation can be prevented by antiplatelet drugs, of which acetyl salicylic acid (Aspirin®) is the oldest and the most widely used. Aspirin is currently used in the secondary prevention of ischaemic cerebrovascular accidents and myocardial infarction. Patients with transient ischaemic attacks, angina pectoris or claudication are at risk for acute thrombo-embolic complications and they also benefit from regular aspirin intake. Anti-platelet therapy reduces the risk of vascular death by about one sixth and the risk of non-fatal myocardial infarction or stroke by about one third in patients with unstable angina, suspected acute infarction or a past history of myocardial infarction, stroke or transient ischaemic attack.[1-3] Apart from aspirin, other antiplatelet drugs such as ticlopidine were also shown to be beneficial.

Increased platelet activation may also be related to the complication of early rethrombosis after coronary or peripheral thrombolysis. On the other hand, a disturbed platelet function may cause part

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Blood Reviews (1995) 9, 143-156

of the bleeding problems associated with therapeutic thrombolysis. Bleeding complications indeed also occur with the newer fibrin-specific thrombolytic agents such as t-PA, which cause only a slight decrease of fibrinogen levels.

Platelet Ultrastructure

Resting platelets circulate as discoid anuclear cells, originating from megakaryocytes in the bone marrow. They are about 3 μm in diameter and 1 μm thick; hence they are the smallest blood cells. The platelet is surrounded by a typical bilamellar plasma membrane that extends through the multiple channels of the surface connected canalicular system, greatly increasing the surface area of the platelet. The plasma membrane is composed of phospholipids; the negatively charged phosphatidylserine and phosphatidylinositol residues are primarily confined to the cytoplasmic side, where they may serve as substrates for phospholipases (see below). Through this phospholipid bilayer, intrinsic glycoproteins such as glycoprotein (GP) IaIIa, GP Ib, GP IIbIIIa and GP IV are extruding, serving as platelet receptors for activating and inhibitory agents. Their intracytoplasmic tails are in close contact with elements of the intraplatelet contractile system, e.g. the linkage between GP Ib and actin-binding protein[4] and of GP IIbIIIa and actin filaments.[5]

The platelet cytoskeleton is composed primarily of actin filaments; actin constitutes 15–20% of the total platelet protein content. The organization of actin filaments is maintained by their association with proteins such as tropomyosin, α-actinin and actin-binding protein. Upon platelet activation, myosin associates with the actin filaments, thus generating the tension required for the centralization of granules.[6] In addition to the cytoplasmic actin filaments, platelets contain a membrane skeleton, composed of short actin filaments crosslinked by actin binding proteins and connected to the plasma membrane, primarily through GP IaIIa and GP Ib. This membrane skeleton stabilizes the lipid bilayer and regulates the shape of the plasma membrane. Apart from actin and actin binding proteins, the platelet cytoskeleton is composed of a microtubular coil, just beneath the platelet membrane. This microtubular coil, composed of tubuline, is involved in maintaining the discoid shape of the unstimulated platelet.[7]

The dense tubular system is the equivalent of the smooth endoplasmic reticulum in other cells; it is the site where calcium is sequestered and where enzymes involved in prostaglandin synthesis are localized. It lies in close contact with the channels of the open canalicular system, forming a membrane complex.

There are numerous organelles dispersed in the cytoplasm, including (few) mitochondriae, glycogen particles, lysosomes and peroxisomes. α granules and dense granules are platelet-specific storage granules. α granules contain mainly proteins such as platelet factor 4, β-thromboglobulin, platelet derived growth factor, fibrinogen, fibronectin, thrombospondin, plasminogen activator inhibitor I and vWF. Dense bodies are rich in serotonin, adenosine diphosphate (ADP) and calcium. Plow and Collen[8] have shown that α granules contain small amounts (62 ± 24 ng/10^9 platelets) of an antigen, immunochemically indistinguishable from plasma α_2-antiplasmin. Although this platelet α_2-antiplasmin constitutes only 0.05% of the blood level and only 1/10 000 of platelet proteins, local concentrations in fibrin/platelet clots could however approach significant levels.

Upon platelet activation, platelets lose their discoid shape, become relatively spherical in form and extend long, spiky pseudopods as well as bulky surface protrusions. The organelles are contracted towards the platelet center and are enclosed by a tight-fitting ring of newly-reassembled microtubules and microfilaments. Finally, the contents of the secretory organelles are extruded. During secretion, granule membranes fuse with those of the surface connected canalicular system, with the diffusion of internal granular membrane proteins such as P-selectin (GMP-140 or PADGEM) into the plasma membrane. Whereas dense body contents are easily secreted, α granule release requires higher agonist concentrations, while lysosomal granule secretion only occurs with powerful activating agents such as thrombin or high doses of collagen. In addition to the contents of the three types of granules, platelets will also produce and secrete pharmacologically active substances such as thromboxane $(Tx)A_2$ and platelet activating factor (PAF) (in the case of strong agonists such as thrombin and high dose collagen) during their activation and aggregation, establishing a positive feedback system.

Mechanisms of Platelet Activation

The Figure is an attempt to visualize in a schematic way the different platelet membrane receptors and pathways leading to platelet activation or inhibition.

Platelet Receptors

Platelets can be activated by a variety of physiological (thrombin, TxA_2, collagen, ADP, PAF, serotonin, epinephrine...) and pharmacological agents (the calcium ionophores, the cyclic endoperoxide analogues...). All these agonists are believed to exert their effects through the interaction with specific receptors on the platelet plasma membrane. All of the agonist receptors which interact with guanine-nucleotide binding regulatory proteins or G proteins (see further) that have been identified to date, consist of a single polypeptide with an extracellular N-terminal domain which serves as the activator-binding domain, seven hydrophobic transmembrane domains and an intracellular C-terminal domain which is in connection with cytoplasmic second

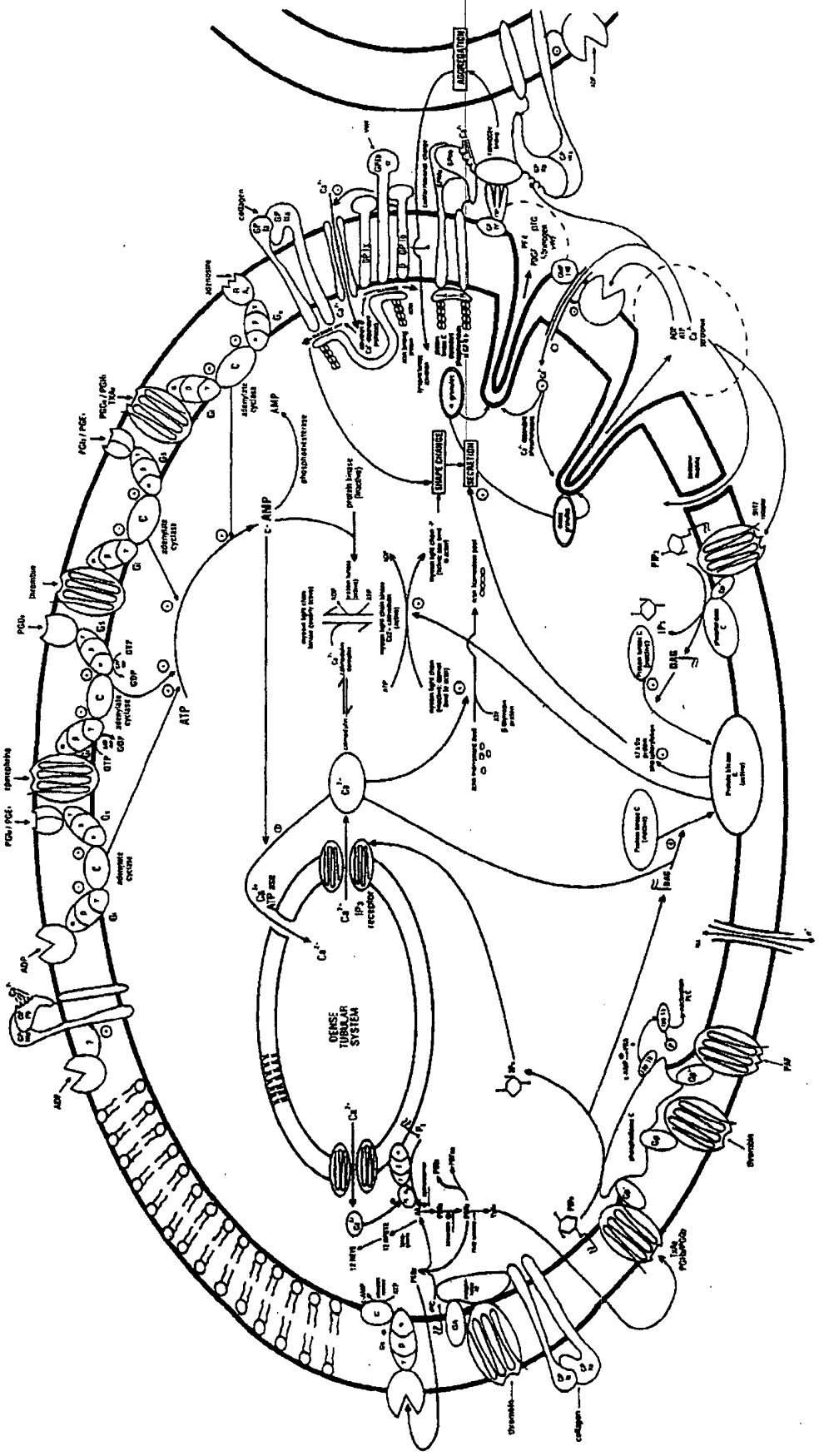

Fig. 1 Schematic representation of receptors and pathways leading to platelet activation and inhibition. AA: arachidonic acid; ADP: adenosine diphosphate; ATP: adenosine triphosphate; C: catalytic unit of adenylate cyclase; Ca2+: calcium; (c-)AMP: (cyclic-) adenosine monophosphate; DAG: diacyl glycerol; G: guanine-nucleotide binding regulatory protein (GA, Gp, Gp', Gp", Gp"'); GDP: guanosine diphosphate; Gi: inhibitory G-protein; GP: glycoprotein; Gs: stimulatory G-protein; GTP: guanosine triphosphate; HETE: hydroxyeicosatetraenoic acid; HPETE: hydroperoxyeicosatetraenoic acid; IP3: inositol triphosphate; kDa: kilo Dalton; PAF: platelet-activating factor; PDGF: platelet derived growth factor; PF4: platelet factor 4; PG: prostaglandin; PIP2: phosphatidyl inositol biphosphate; PKA: protein kinase A; PLA2: phospholipase A2; TG: thromboglobulin; TSP: thrombospondin; TxA2: Thromboxane A2; vWF: von Willebrand factor.

messenger generating enzymes. The primary effects of these agonists are often enhanced by secondary effects attributable to the synthesis of TxA_2 from released arachidonic acid (AA) and to the secretion of ADP. The best known platelet receptors are further described (see also the table).

vWF Receptor. vWF is synthesized in megakaryocytes and endothelial cells and is also found in platelet α-granules, plasma and subendothelium. Mature vWF has a molecular weight of 260 kilo Dalton (kDa) (2050 amino acids) and consists of multimers, ranging from 500 kDa (dimers) to over 10 000 kDa. The largest multimers, which are the most effective in promoting platelet adhesion and aggregation, are present in the subendothelium and in platelets but are only transiently observed in normal blood.[9] Upon binding to subendothelial components, especially type VI collagen, vWF undergoes a conformational change which enables it to bind to platelet GP Ib. In vitro, this conformational change can be induced by addition of ristocetin or botrocetin; in vivo, high shear forces may induce the required conformational change of vWF (see further).

GP Ib is one of the major sialoglycoproteins of the platelet membrane; it is lacking in the platelets of patients with Bernard-Soulier syndrome. GP Ib consists of two disulfide-linked subunits, GP Ib α (143 kDa) and GP Ib β (22 kDa). There are about 25 000 molecules of GP Ib per platelet. GP Ib is noncovalently bound to GP IX in a 1:1 ratio. The binding sites for vWF and thrombin (see further) are located in the N-terminal portion of the α-chain. The intracytoplasmic tail of the β-chain can be phosphorylated.

The interaction between vWF and GP Ib results in platelet activation and in the generation of an intraplatelet signal necessary to activate GP IIb/IIIa,[10] which in turn will bind vWF through the tripeptide sequence arginine-glycine-aspartic acid (RGD) in the C-terminal of vWF. This binding leads to platelet spreading and irreversible platelet adhesion, which can resist the high shear forces in circulating blood, and also to platelet aggregation (see further).

Thrombin Receptor. Thrombin is a very powerful platelet stimulus, causing shape change, aggregation

Table 1 Platelet receptors

a. G-protein-linked platelet activating receptors

Receptor	*Agonist(s)*	*Molecular weight*	*Number of receptors/platelet*	*Main receptor-coupled enzyme*
Thrombin receptor	thrombin	47 kDa	1700–1800	PLC+, Adcyc−
α_2 receptor	epinephrine	64 kDa	200–300	Adcyc−, prot.kin C+
$5HT_2$ receptor	5-OH tryptamine	232 kDa	20–2500	PLC+
TxA_2 receptor	TxA_2, PGH_2, PGG_2	37 kDa	1200	PLC+, Adcyc−
PAF-receptor	PAF	220 kDa	150–2000	PLC+
V_1A-receptor	vasopressin	125 kDa	170	PLC+

b. G-protein-linked platelet inhibiting receptors

Receptor	*Agonist(s)*	*Number of receptors/platelet + affinity constant*
A_2 receptor	adenosine	5500 (KD 0.16 μM) 22 000 (KD 2.9 μM)
PGI_2 receptor	PGI_2, PGE_1	100–3000 (KD 10 nM) >3000 (KD 1 μM) 1410 (KD 60 nM)
PGD_2 receptor	PGD_2	200–800 (KD 10 nM)

c. Platelet glycoproteins

Receptor	*Agonist(s)*	*Molecular weight*	*Number of receptors/platelet*
GP Ib/IX	vWF	143α–22β/22 kDa	25 000
GP IaIIa ($\alpha_2\beta_1$)	collagen	153/130 kDa	1000–2000/7000
GP Ic/IIa ($\alpha_5\beta_1$)	fibronectin	160/130 kDa	?/7000
GP Ic'/IIa ($\alpha_6\beta_1$)	laminin	160/130 kDa	?/7000
GP IIbIIIa ($\alpha_{IIb}\beta_3$)	fibrinogen (+vWF, fibronectin, vitronectin)	125α–22β/95 kDa	40 000–80 000
($\alpha_v\beta_3$)	vitronectin, thrombospondin	195/95 kDa	1500
GP IV (or IIIb)	thrombospondin, collagen	88 kDa	12 000–19 000
GP VI	collagen	62 kDa	?

d. Ill-defined receptors

Agonist	*Receptor characteristics*
ADP	P_{2T} type purine receptor, one or two receptors involved; Adcyc−
PGE_2	linked to prot.kin C?

Abbreviations: PLC: phospholipase C; Adcyc: adenylate cyclase; prot.kin C: protein kinase C; +: stimulation; −: inhibition

and secretion from dense granules, α granules and lysosomes. Its intermediate affinity receptor (approximately 1700–1800 copies/cell) was described in 1991, from the work of Vu et al[11] and Rasmussen et al.[12] It consists of a N-terminal extraplatelet domain, seven transmembrane domains and an intracytoplasmic C-domain. Thrombin cleaves its own receptor between arginine 41 and serine 42. It was proposed by Vu et al[11] that the new N-terminal sequence forms a tethered ligand capable of activating the receptor. This was confirmed by other investigators, using synthetic oligopeptides. The finding that cleavage of the receptor activates it, makes the thrombin receptor probably the first of a new series of cleavage-induced receptors. Other proteases such as trypsin, cathepsin G or plasmin may also be capable of cleaving their receptor and hence activate platelets.

Subsequent to thrombin activation, desensitization to further thrombin activation occurs with internalization of the thrombin receptors in endosomes. From the endosomes, almost three quarters of the receptors are transferred to lysosomes and degraded.[13] Desensitization occurs through phosphorylation of serine and threonine residues. It is not yet known which kinase(s) are responsible: protein kinase C, the β-adrenergic receptor kinase or c-AMP dependent protein kinase.[13] Resensitization occurs in two phases, a first one during the first 30 min and a slow one taking about 20 h and based upon de novo receptor synthesis.[14] There are no arguments to suggest that a second thrombin receptor exists. Thrombin binds to GP Ib with high affinity but this interaction does not evoke a functional response,[15] apart from accelerating activities. Following thrombin receptor activation, G protein- coupled receptors become phosphorylated and phospholipase C is activated (see further).

Collagen Receptor. Platelets adhere to the connective tissue protein collagen with resulting shape change and dense granule release; adhesion is partly and aggregation is largely dependent on ADP and prostaglandin (PG)H_2/TxA_2 released.[16,17] The quaternary structure of collagen, with the triple helix of three polypeptide chains and the polymerisation of these monomers into fibers, is crucial in the activation of platelets. Platelet adhesion to collagen is enhanced by magnesium, and probably also involves adhesive proteins such as vWF and fibronectin. Receptor occupancy is associated with phospholipase C activation and an increase in intracellular calcium when using aequorin as intracellular calcium detector. There are several candidates for the function of collagen receptor, e.g. GP Ia/IIa, GP IV and GP VI. As collagen is a filamentous molecule with repeating subunits, it may interact simultaneously with multiple sites on the platelet surface.

GP Ia (integrin α2) is a large transmembrane single chain glycoprotein (153 kDa), interacting at its cytoplasmic side with actin-binding protein. Nieuwenhuis et al[18] described a patient with a lifelong history of bleeding, with platelets deficient in this glycoprotein. Platelet aggregation and secretion induced by collagen did not occur, while adherence of platelets to subendothelium in a flowing blood stream was especially defective in platelet spreading. Platelets from another patient with an autoantibody against GP Ia also did not respond to collagen.[19]

Attempts to purify the collagen receptor yielded a heterodimer of GP Ia with the integrin GP IIa (β1). Studies with monoclonal antibodies against this complex have shown that collagen binds directly to GP Ia/IIa and indirectly to GP IIb/IIIa (αIIbβ3) via vWF and fibronectin.[20] It has been shown that tyrosine kinase becomes activated upon collagen induced activation, but so far, no conclusive explanation for the collagen dependent activation system has been offered.

ADP Receptor. Purine receptors are classified as P_1 (which recognize adenosine) and P_2 (which recognize ADP and adenosine triphosphate (ATP)); the platelet ADP receptor is a P_{2T} type purine receptor.[21] In contrast to other P_2 receptors, ATP is an antagonist to ADP on platelets, which makes the platelet ADP receptor unique. Estimates of the number of receptors per platelet range from 400–1200 to 18 500, depending on the method used(radioligand binding studies with β(32^P)2-methylthioADP[22] versus (^{3}H) ADP using formaldehyde-fixed platelets[23]). Unlike the other receptors discussed here, the ADP receptor has not yet been identified, but there is good reason to believe that it might be a 43 kDa protein.[24]

In vitro, ADP causes shape change, aggregation and the release of granule contents. This release is only observed with subphysiological extracellular calcium levels (not with heparin or with ethylene diamino tetraacetate (EDTA), but with citrate as anticoagulant) and is a consequence of prostaglandin synthesis caused by platelet-platelet contact.[25] ADP plays a role in secondary aggregation induced by other aggregating agents. ADP also inhibits adenylate cyclase, possibly through another receptor,[26] or through two different effector mechanisms associated with one receptor[27] (Gi for inhibition of adenylate cyclase, other G protein(s) for its other actions?). Colman's hypothesis of two separate ADP receptors relies on his experiments with 5'-p-fluorosulfonylbenzoyl adenosine (FSBA), which inhibits ADP-induced shape change but not the increase in intracellular calcium nor ADP's action on adenylate cyclase. Doubt has however risen to the specificity of FSBA. FSBA binds to a protein of 100 kDa (2500 sites per platelet), called 'aggregin' which may be closely associated with the GP IIb/IIIa-fibrinogen receptor complex, hindering its assembly until ADP is bound.[28]

Epinephrine (Adrenaline) Receptor. Epinephrine (Adrenaline) only induces platelet aggregation in the presence of subphysiological calcium concentrations,

as occurs in citrated plasma.[29] Aggregation as monitored in the light transmission aggregometer occurs without preceding shape change (disc to sphere transformation). Probably epinephrine induced aggregation is dependent on trace amounts of ADP in the medium.[30]

Platelets have stimulatory α-2 adrenoceptors and inhibitory β adrenoceptors; in most individuals the α-2 adrenoceptors predominate. The α-2 adrenoceptor is coupled via G proteins to adenylate cyclase (in contrast to α-1 adrenoceptors which are linked to phospholipase C, and which may be present in about 30% of individuals.[31] In view of the fact that inhibition of adenylate cyclase in itself is not sufficient to cause platelet aggregation, and as a result of studies with synthetic analogues, it was hypothesized that there may be two different α-2 adrenoceptors, one mediating aggregation and one inhibiting adenylate cyclase,[32] in analogy with the ADP receptor. Recently, Nieuwland et al[33] demonstrated that α_{2A}-adrenergic receptors activate protein kinase C via a G-protein of the Gi-family, independent of the phospholipase C pathway and DAG formation. This activation resulted in stimulation of the Na^+/H^+ exchange, exposure of fibrinogen binding sites on the GP IIb/IIIa complex, and aggregation.

In 1986, the (unique) α-2 adrenergic receptor (present in 200–300 copies per platelet) has been purified to apparent homogeneity as a single polypeptide, with a molecular weight of 64 kDa.[34] The DNA was sequenced; the gene codes for a 450 amino acid protein with seven clusters of hydrophobic residues, representing the membrane spanning domains, as is characteristic for a guanosine triophosphate (GTP) linked receptor. The amino terminal lies extracellularly, the carboxy terminus intracytoplasmic.[35] After prolonged occupancy of the α-2-receptor with epinephrine, a time-dependent decrease or 'desensitization' of subsequent aggregation responses to epinephrine is seen, by a mechanism that is incompletely understood.[36]

The concentrations of epinephrine required to induce aggregation are in the micromolar concentration range, far in excess of the nanomolar concentrations present in the circulation. This suggests that aggregation induced by epinephrine is unlikely to have any significance in vivo, especially because it only occurs at lowered extracellular calcium concentrations (with citrate as anticoagulant); however epinephrine may sensitize platelets to other platelet agonists.[30,37]

Serotonin or 5-Hydroxytryptamine (5-HT) Receptor. The platelet 5-HT_2 receptor is coupled to phospholipase C through a G protein, and receptor activation results in rapid phosphoin sitide breakdown, increase in intracellular calcium levels and appropriate protein phosph rylation. The human serotonin 5-HT_2 receptor has been cloned and was shown to have the classical seven transmembrane domains.[38] Aggregation is inhibited specifically by a selective 5-HT_2 antagonist such as ketanserin.[39] Apart from being a platelet agonist, serotonin is also rapidly taken up by passive diffusion and by a high affinity energy-dependent carrier, and released with the secretion of dense granules.[40]

TxA_2 and Cyclic Endoperoxide Receptor. Free AA is metabolized by platelets via the prostaglandin endoperoxides PGG_2 and PGH_2 to the potent TxA_2. AA is released from the membrane phospholipids by phospholipases activated by strong platelet agonists such as thrombin or collagen and by the aggregation process itself. PGH_2 has a half-life of about 5 min, TxA_2 is broken down to the inactive TxB_2 with a half-life of less than 1 min. TxA_2 causes shape change, aggregation and release of granule contents and acts via membrane receptors coupled by a G protein to phospholipase C and probably also to a calcium channel. Binding studies with the radioactive cyclic endoperoxide analogue (^3H) U 46619 provided evidence for two TxA_2/PGH_2 receptors: one with higher affinity (dissociation constant (K_D) 41 nM, 1166 sites per platelet) related to shape change and protein phosphorylation, and another one with low affinity (K_D 1.46 μM) related to the release reaction.[41] Takahara et al[42] and Dorn II and DeJesus[43] also presented evidence for the existence of two separate TxA_2/PGH_2 receptors (or functionally distinct forms of a single site), one mediating shape change and another mediating platelet aggregation. In the same year, the receptor protein was sequenced (molecular weight 37 kDa), and hydrophobicity analysis revealed seven hydrophobic sequences that could represent transmembrane regions consistent with a G protein coupled receptor,[44] linked to phospholipase C. This G protein became known as Gq. Post-translational modifications or sequence polymorphism in the gene sequence may modify its affinity for natural ligands.[45]

PAF Receptor. PAF is formed in certain cells, including platelets, from the membrane phospholipid 1-O-alkyl-2-acyl-sn-glyceryl-3-phosphorylcholine by the consecutive actions of phospholipase A_2 and acyltransferase. PAF is released from platelets following stimulation by the calcium ionophore A 23187, thrombin or collagen but not by ADP, AA or PAF itself. PAF release has been suggested to be the 'third pathway' through which platelet activation may be amplified, in addition to ADP release and AA metabolism.[46] PAF causes shape change and aggregation, but in human platelets, PAF is dependent on AA metabolism to cause granule contents release (and hence it is not likely to be a major pathway in human platelets). PAF induces inositol phospholipid metabolism and calcium mobilization. (^3H)PAF has been shown by several authors to bind specifically and with high affinity to human platelets, although estimates of the K_D range from 0.053 to 37 nM and receptor numbers from 150 to 1983 sites per platelet.[47] The receptor, which is G protein coupled, has an

apparent molecular weight of 220 kDa.[48] It has been cloned and was shown to have the characteristic structure in which a single polypeptide chain crosses the plasma membrane seven times.[49] PAF internalization occurs during platelet activation and is probably related to the reorganization of the plasma membrane.

Vasopressin Receptors. Human platelets possess V_1 vasopressin receptors which mediate phosphoinositol metabolism, an increase in cytosolic free calcium and platelet aggregation.[50,51]

Adenosine Receptor. Adenosine inhibits shape change, aggregation and release induced by all platelet agonists. These inhibitory actions are mediated by an adenosine receptor of the A2 subtype that stimulates adenylate cyclase via a Gs regulatory protein. Adenosine can generate up to a 10-fold increase in platelet cyclic adenosine monophosphate(c-AMP) content. Studies with (^{3}H) 5'N-ethylcarboxamidoadenosine (NECA) revealed the existence of high affinity binding sites (K_D 0.16 μM, 5500 sites per platelet) and low affinity binding sites (K_D 2.9 μM, 22 000 sites per platelet).[52] Adenosine is taken up by platelets, phosphorylated by an adenosine kinase to adenosine monophosphate (AMP) and further phosphorylated to ADP and ATP, entering the metabolic nucleotide pool.

PGI_2/PGE_1 Receptor. Several studies have shown that PGI_2 and PGD_2 have their own separate receptors on platelets (there may also be a weak agonist interaction of PGD_2 with the TxA_2 receptor), while PGE_1 acts via the PGI_2 receptor.[53] There are two binding sites on the PGI_2/PGE_1 receptor, one with high affinity (K_D 10 nM) but small numbered (100–3000 per platelet) and a more abundant one (3000 or more per platelet) with low affinity (K_D 1 μM).[54] Other authors have detected only one (high affinity) binding site (K_D 60 nM, 1410 sites per platelet).[55] A PGI_2/PGE_1 binding protein with the characteristics of the receptor has been isolated and purified from human platelet membranes. It consists of two subunits of 85 and 95 kDa.[56] The receptor is coupled via the Gs regulatory protein to the adenylate cyclase enzyme.

PGD_2 Receptor. There is only one class of binding sites for PGD_2 on the platelet membrane, with a K_D value of about 10 nM. There are 200–800 PGD_2 binding sites per platelet.[57–59] The receptor is coupled via the Gs regulatory protein to adenylate cyclase, thereby increasing c-AMP concentrations. Platelets from some patients with myeloproliferative disorders fail to respond to PGD_2 but do retain sensitivity to PGI_2.[60]

PGE_2 Receptor. PGE_2 has weak, rather complex actions on platelets and can inhibit or potentiate platelet activation depending on the conditions. It was suggested that the weak inhibitory effects of PGE_2 are mediated via the PGI_2/PGE_1 receptor, but Eggerman et al[61] proposed the existence of a separate PGE_2 receptor, to which PGE_1 might also bind. Recently, evidence was shown for the hypothesis that PGE_2 potentiates platelet aggregation by priming protein kinase C,[62] while Kunapuli et al[63] cloned and expressed a prostaglandin receptor from human erythroleukaemia cells which inhibited c-AMP formation in response to PGE_2, which was bound with high specificity.

Coupling of Receptor Occupancy to the Generation of Second Messengers

Guanine Nucleotide Binding Regulatory Proteins and Effectors. Numerous cell receptors for platelet agonists (and antagonists) are coupled via guanine nucleotide- binding regulatory proteins or G proteins to second messenger generating enzymes. These enzyme systems are adenylate cyclase, phospholipase C and phospholipase A_2. G proteins are composed of 3 subunits, of which the α subunit is the guanine nucleotide binding site and which can be in some cases ADP-ribosylated by cholera toxin or pertussis toxin. In many cases, the α subunit is responsible for the interaction between receptors and effectors. The heterodimer proteins β and γ help to anchor the G protein to cell membranes. The activation of ion channels, the inhibition of adenylate cyclase and the activation of phospholipase A_2 are also mediated by the β-γ subunit. The α subunit is specific, the β-γ dimer is similar or identical in the different proteins. At present, 22 forms of G α, 4 forms of G β and 7 forms of G γ have been described, yielding hundreds of possible combinations.[13] On binding a GTP molecule, the heterodimer β-γ dissociates from the α subunit, resulting in activation of the second messenger-generating enzyme by the α-GTP subunit. The intrinsic GTPase activity of α GTP terminates this interaction and the α GDP formed reassociates with β-γ.[64]

Adenylate cyclase uses ATP to form c-AMP and is activated by a Gs regulatory protein and inhibited by a Gi regulatory protein. In platelets, the former one is stimulated by adenosine, PGI_2, PGD_2 and PGE_1 with an increase in c-AMP levels as a result. Gi is stimulated by thrombin, ADP and epinephrine with a resulting decrease in c-AMP levels. Gs α may be ADP-ribosylated by the cholera toxin which results in continuous activation of adenylate cyclase. Gi is a substrate for the pertussis toxin, which blocks the Gi-mediated suppression of c-AMP formation induced by thrombin, ADP or epinephrine. The β-γ dimer appears to mediate most of the inhibition of adenylate cyclase caused by α 2-adrenergic agonists.[64] The Gs and Gi regulatory proteins may also control channel activity: Gs may inhibit Na+ channels and stimulate dihydropyridine-sensitive Ca2+ channels; Gi may stimulate K+ channels and inhibit Na+ channels.[65]

Phospholipase C catalyses the breakdown of phosphatidylinositol(4,5)biphosphate (PIP_2) into diacylglycerol (DAG) and inositol(1,4,5)triphosphate (IP_3). DAG activates protein kinase C and IP_3 induces a release of Ca^{2+} from the dense tubular system (see further). Phospholipase C is stimulated by a G protein, referred to as Gp. However, there is probably more than one G protein involved, since platelets contain G proteins capable of activating phospholipase C some of which are a substrate for the pertussis toxin (formerly thought to be Gi) and others are not. For instance, thrombin induced phosphoinositide hydrolysis is inhibited by pertussis toxin, but U 46619 induced hydrolysis is not.[66] C-AMP and cyclic-guanosine monophosphate (c-GMP) both inhibit phospholipase C, probably by an action at the G protein level.

Phospholipase A_2 liberates AA from the dense tubular system and the plasma membrane. AA is further reconverted to TxA_2 (see further). Phospholipase A_2 is activated by a rise in cytosolic calcium (due to phospholipase C induced IP_3 synthesis), but probably also through a direct activation by G $\beta\gamma$.

Second Messengers Involved in Platelet Activation and Inhibition.

1. IP_3 and Calcium. The seven membrane-spanning receptors of epinephrine, serotonin, PAF, TxA_2 and thrombin are coupled through the already discussed G proteins to phospholipase C γ 1 which hydrolyses PIP_2 to give both IP_3 and DAG. In other cell types, phospholipase C γ 1 can be activated by tyrosine kinase-linked receptors, such as the platelet derived growth factor receptor, without involvement of G-proteins.[67] Phospholipase C γ has recently also been detected in the cytosol of human platelets.[68] IP_3 binds to an IP_3 receptor to mobilize stored calcium and to promote an influx of external calcium.[69] The IP_3 receptor of the dense tubular system contains typical membrane-spanning domains in the C-terminal region which anchor the protein in the membrane with four of the subunits combining to form the functional IP_3-sensitive calcium channel. The large N-terminal domain lies free in the cytoplasm with the IP_3 binding site located at its end, at distance from the channel-forming C-terminal region.[70] Calcium channels in the platelet outer membrane also seem to be regulated by IP_3, together with IP_4.

Calcium mobilization leads to activation of phospholipase A_2, which is followed by TxA_2 formation (see further). Na+/H+ exchange leading to an increase in intracellular pH is required for this calcium mobilization[71] and for phospholipase A_2 activation.[72] ADP-induced calcium influx, unlike that induced by other agonists, occurs without measurable delay and therefore appears to be due to activation of receptor-operated calcium channels rather than to IP_3-mediated processes.[73]

All known platelet agonists increase the intraplatelet calcium concentration, as measured with the aequorin method. Epinephrine- and collagen-induced calcium elevations cannot be detected with the intracellularly-trapped fluorescent indicator quin-2, probably due to the lower sensitivity, the higher buffering capacities and the inability of quin-2 to detect local calcium increases. An increase in intracellular calcium leads to the activation of the calmodulin-regulated myosin light chain kinase, and hence to phosphorylation of myosin and to shape change (see further). When platelet activation is induced by a calcium ionophore such as ionomycin (which does not activate phospholipase C), an internal Ca^{2+} threshold of 0.5 μM for shape change, 0.8 μM for serotonin release and 2 μM for aggregation were found using the quin-2 method. Agonists such as thrombin or the prostaglandin endoperoxide analogue U 44069 which induce activation of phospholipase C and protein kinase C can evoke shape change and secretion at near basal internal Ca^{2+} levels.[74,75]

2. Diacylglycerol (DAG). The second product formed by phospholipase C starting from PIP_2 is DAG. DAG promotes the translocation of protein kinase C from cytoplasm to membrane and activates protein kinase C. Protein kinase C is a 80 kDa widely distributed family of enzymes that transfers phosphate from ATP to the serine or threonine residues of specific proteins in the presence of phosphatidylserine and calcium ions.[76] DAG and its synthetic derivatives like phorbol esters, induce platelet aggregation and serotonin secretion, although with little change in prior platelet shape. A protein with an apparent molecular weight of 47 000, termed P 47 or pleckstrin, is the major platelet substrate of protein kinase C.[77] Its exact identity and function has not yet been clarified, although it may be related to platelet secretion.[78] The other major polypeptide that undergoes phosphorylation by protein kinase C is the 20 kDa light chain of platelet myosin, which is however mainly the substrate of the Ca^{2+} and calmodulin-regulated myosin light chain kinase.[79] There is an apparent synergism between protein kinase C activation and calcium ions, as is shown by the results obtained with the combination of phorbol esters (which only activate protein kinase C) and the calcium ionophore A 23187 (which directly introduces Ca^{2+} into the cytosol). Both activities are required for an optimal platelet response, including secretion. Protein kinase C activation also was shown to block the inhibition of adenylate cyclase by epinephrine.[80]

On the other hand, protein kinase C activation has also some inhibitory effects on platelet function, hence establishing a negative feedback system.

3. Prostaglandins and TxA_2. The principal substrates of phospholipase A_2 in intact platelets stimulated by thrombin are phosphatidylcholine and phosphatidylethanolamine with liberation of AA.[81] Phospholipase

A_2 is stimulated by an increase in Ca^{2+}, as occurs through the action of IP_3, but also through a calcium-independent mechanism, probably involving a direct interaction with a G protein.

AA is enzymatically converted by cyclooxygenase (which can be irreversibly acetylated by acetyl salicylic acid) to the labile prostaglandin cyclic endoperoxides PGG_2 and PGH_2 which by themselves can aggregate platelets. PGH_2 has a half-life of approximately 5 min and is rapidly converted, mainly by thromboxane synthase, to TxA_2. PGH_2 and TxA_2 produced by aggregating platelets play an important role in causing secondary aggregation and secretion and are part of the amplification mechanisms.

PGH_2 can also be converted to the inhibitory PGD_2, to the inactive or minimally inhibitory $PGF_2\alpha$[82] and to PGE_2. PGE_2 can also be released from vascular cells under various stimulations[83,84] and might be produced in excess in some pathological conditions such as diabetes in which thrombosis occurs.[85] Contradictory results on the action of PGE_2 have been published: some authors consider PGE_2 as a platelet stimulatory prostaglandin,[86–89] while others could never detect any stimulatory effect of PGE_2.[52,59,90] As already mentioned, there was a recent report from the Perugia-group that PGE_2 might potentiate platelet aggregation by priming protein kinase C.[62]

The cyclic endoperoxides may also leave the platelet and enter the endothelial cells, where they are converted to the platelet inhibitory and vasodilating PGI_2.[91]

4. c-AMP. The enzyme adenylate cyclase forms c-AMP out of ATP. Increased c-AMP levels lead to a more difficult calcium mobilization and to the sequestration of calcium in the dense tubular system, and hence to platelet inhibition or reversal of platelet aggregation. The dense tubular Ca^{2+}-ATPase responsible for Ca^{2+} sequestration is more active with higher c-AMP levels.[92] Increased c-AMP additionally inhibits PIP_2 hydrolysis and the formation of IP_3, presumably by an effect on phospholipase C. Phospholipase C may lose its ability to be stimulated by platelet agonists through the phosphorylation by a c-AMP-dependent protein kinase and translocation of a 22 kDa protein rap 1-b, with which phospholipase C is closely associated.[93] The activity of adenylate cyclase is regulated by G proteins (see above): it is stimulated by Gs and inhibited by Gi. Gs is coupled to the receptors for the inhibitory prostaglandins PGI_2/PGE_1 and PGD_2 and to the adenosine receptor. Some aggregating agents (thrombin, epinephrine and ADP) inhibit adenylate cyclase via their action on Gi (more specifically the β-γ subunit) and hence reduce levels of c-AMP in platelets with elevated c-AMP concentrations. This phenomenon however does not suffice to result in platelet aggregation. C-AMP is broken down to inactive AMP by the action of phosphodiesterases.

5. c-GMP. Aggregating agents increase levels of c-GMP in platelets, but this is the effect, rather than the cause of platelet aggregation.[94] In fact, c-GMP is inhibitory in platelets, acting as a feedback inhibitor of platelet activation. The synthesis of c-GMP is stimulated by nitric oxide, formed by normal endothelium, and by vasodilators.[95] C-GMP is degraded by phosphodiesterases.

Reactions Leading to Platelet Shape Change and Secretion

The discoid shape of resting platelets is maintained by circumferential bundles of microtubules beneath the platelet membrane and an extensive network of short actin filaments, forming a membrane skeleton.[96] Upon platelet activation, these microtubular bundles depolymerize, while actin polymerization starts, resulting in platelet shape change and the formation of pseudopods. In unstimulated platelets, 40–50% of the total actin is in the filamentous form, in activated platelets 70–80%. Actin polymerization is based upon a shift of actin subunits (Mr 42 000, bound to a polypeptide named β 4 thymosin,[97]) from the monomeric pool to the filamentous pool of actin. Filamentous actin has a low affinity actin-binding pointed end and a high affinity fast growing barbed end. In the resting state, these barbed ends are capped by one of the known capping proteins, such as Cap Z and gelsolin. In these resting platelets, an equal number of ADP-bound monomers are bound per time unit to the barbed ends and are lost from the pointed ends. Upon cell activation, with ATP coming available, assembly of ATP-bound actin monomers at the barbed ends is much more increased, resulting in net actin polymerisation. These processes are regulated by PIP_2 metabolization in at least three ways. First, hydrolysis of PIP_2 produces DAG which increases the actin nucleating activity of an unidentified membrane-bound protein.[98] Secondly, the replenishment of actin monomers with ATP is regulated by profilin, a protein which is released from its membrane binding site with PIP 2 hydrolysis.[99] Binding of profilin to PIP_2 is also thought to prevent the binding of certain phospholipases C to PIP_2. Upon tyrosine phosphorylation of phospholipase C, it now can displace profilin. Thirdly, the activity of certain capping proteins such as Cap Z is also regulated by the presence of inositol phospholipids.[100]

Actin filaments make contact with the platelet membrane at so-called local adhesion sites. These are specialized membrane domains where the actin cytoskeleton is anchored to transmembrane integrin molecules, such as GP IIbIIIa[101] or GP IaIIa, but also to the non-integrin molecules GP IbIX. There are, however, two major differences between GP IIbIIIa on the one hand and GP IbIX and GP IaIIa on the other hand in regard to the attachment to the platelet cytoskeleton. First, GP IbIX and GP IaIIa are connected to the actin

network in resting platelets. The association between these glycoproteins and the membrane skeleton is disrupted during the early stages of platelet activation, resulting in loss of vWF binding to GP Ib. GP IIbIIIa in contrast is connected only once platelets start to aggregate. Secondly, GP IbIX and GP IaIIa bind to the microfilaments of the membrane skeleton (see above), while GP IIbIIIa becomes attached with cytoplasmic actin filaments.

A number of recently discovered actin binding proteins are concentrated at the cytoplasmic aspect of these focal adhesions, amongst them: talin or P 235, tensin, vinculin, fimbrin, α-actinin and the tyrosine kinase pp 125FAK. This newly identified tyrosine kinase pp 125FAK or focal adhesion kinase is activated through thrombin-stimulated phosphorylation[102] and hence may play a role in the thrombin-induced shape change. The stability of the focal adhesions is regulated by a small GTP binding protein, Rho,[103] which is a member of the Ras family.

Shape change induced by calcium ionophores such as ionomycin does not occur below an internal Ca2+ concentration of about 300 nM, whereas thrombin, vasopressin, PAF, U 44069 and ADP all cause shape change with little or no increase in internal Ca^{2+}.[104] This discrepancy is due to the role of calcium-independent pathways (e.g. protein kinase C activation) involved in platelet activation induced by the latter agonists.

Platelet granule secretion requires fusion of granular membranes with the plasma membrane or with membranes of the surface connected open canalicular system. Fusion of membranes may be mediated by DAG.[105] Ca^{2+}i and DAG act as coordinate second messengers in the platelet secretory response. Contractile forces within platelets needed for granule centralization and secretion are generated by the interaction of the actin filaments with myosin heads. In activated platelets, myosin surrounds centralized granules, while in unstimulated platelets, it is uniformly distributed throughout the cytoplasm. Platelet myosin consists of two heavy chains and four light chains. The two heavy chains form two globular heads at one end of the molecule, which contains the actin binding sites. Upon platelet activation, myosin monomers may polymerize into filaments when myosin light chain kinase is activated (due to DAG-induced protein kinase C activation and as a result of IP_3 induced intracellular calcium increases with activation of the Ca^{2+}/calmodulin dependent myosin light chain kinase) and the light chain (20 kD) of myosin is phosphorylated.[106] This phosphorylated form can interact with actin with resulting activation of its ATPase activity.[107] The myosin head undergoes a conformational change and the actin and myosin filaments slide past each other, resulting in the generation of contractile force. This interaction of actin and myosin is regulated by the proteins caldesmon and tropomyosin. Tropomyosin has an inhibitory effect on myosin-actin binding and is found concentrated in filopodia of activated platelets. A transient 20 kDa myosin light chain phosphorylation induced by ADP, collagen or a synthetic prostaglandin endoperoxide analog precedes slightly the shape change response (t 1/2 1.5 s versus 2.5 s) and has the same dose-response curve as the shape change phenomenon.[108] Dephosphorylation occurs more slowly, while the platelets can regain their discoidal form.

Activation by platelet agonists such as thrombin and collagen is associated with re-orientation of negatively charged phospholipids such as phosphatidylserine—the so-called flip-flop phenomenon—providing a procoagulant surface required for optimal activation of coagulation factors X and II.[109]

Reactions Leading to Platelet Adhesion and Aggregation

When a blood vessel is damaged, the formation of a hemostatic plug is needed to minimalize blood loss. Platelets are responsible for the initial repair mechanisms. Whenever the subendothelium is exposed due to endothelial damage, platelets will adhere to subendothelial microfilaments and subsequently spread. The most important subendothelial structures next to collagen are fibrinogen, fibronectin and collagen-bound vWF, to which platelets can adhere with their GP IIbIIIa, GP IcIIa or GP αvIIIa and GP Ib receptors. Most of these adhesive proteins contain the RGD sequence as their cell recognition site.[110] The binding of vWF to GP Ib does not involve the RGD sequence, the binding of GP IIbIIIa to vWF—in the absence of fibrinogen—does. Subsequent to adhesion, platelet spreading occurs with additional binding of vWF and fibronectin to GP IIbIIIa.

GP IIbIIIa is a heterodimeric transmembrane molecule composed of an α subunit (GP IIb) and a β subunit (GP IIIa), which are products of different genes. It belongs to the integrin family, where it is known as αIIbβ3. It is absent or defective in Glanzmann's thrombasthenia. The α subunit consists of an extracellular 125 kDa heavy chain and a 22 kDa light chain which crosses the cell membrane. The β subunit is a single chain, 95 kDa protein which, like the α light chain, crosses the platelet membrane.[111,112] There are about 40 000–80 000 GP IIbIIIa molecules at the platelet surface, additional complexes are located in the open canalicular system and in the α granules. Upon platelet activation, these intraplatelet GP IIbIIIa complexes are exposed at the cell surface on α granule fusion with the outer membrane.

The fibrinogen binding site, which can also bind vWF, fibronectin and vitronectin, is located on the NH2 terminal (extracellular) portion of the α and β subunits, which seem to form a three-dimensional ligand binding pocket.[113] Fibrinogen binds in a calcium dependent way to GP IIbIIIa through two RGD sequences located both in the A α chain and

through a sequence of 12 amino acids at the carboxy terminus of the γ chain. RGD-containing snake venoms or synthetic RGD peptides interfere with this binding, inhibiting platelet aggregation.

Immobilized fibrinogen can bind to resting GP IIb IIIa, this is without prior platelet activation,[114] as does immobilized vWF to GP Ib (binding of soluble vWF to GP Ib requires a prior conformational change of vWF, as is seen with the addition of e.g. ristocetin).

Since fibrinogen is a symmetrical molecule, platelet aggregation occurs through the formation of fibrinogen (and/or vWF) bridges between GP IIbIIIa molecules of adjacent platelets. Binding of soluble fibrinogen to GP IIbIIIa however requires prior activation of platelets with the transition of the inactive GP IIbIIIa molecules in resting platelets into activated GP IIbIIIa molecules capable of binding fibrinogen. The GP IIbIIIa activation process probably requires a conformational change of the extracellular parts of the α and β chains. How platelet activation leads to this conformational change is not completely known. It seems logical that the intraplatelet domains of the α and/or β chains of GP IIbIIIa are involved. Phosphorylation of the cytoplasmic domain of GP IIIa was observed by Hillery et al.[115] It has been shown that protein kinase C activation is functionally coupled to exposure of the fibrinogen receptor on GP IIbIIIa.[116] However, platelet agonists which do not activate protein kinase C (since they do not activate phospholipase C) such as ADP or epinephrine (adrenaline) are also capable of inducing these fibrinogen receptors. Increasing intracellular calcium levels can also provoke the necessary conformational change of GP IIbIIIa. C-AMP and c-GMP can downregulate the receptor exposure on GP IIbIIIa, probably by phosphorylation of serine/threonine residues of a not yet identified regulatory intermediating protein.

Recent investigations point to the role of early protein tyrosine kinases and/or tyrosine phosphatases as possible link between platelet activation and aggregation.[117] On the other hand, fibrinogen binding to GP IIbIIIa also evokes certain intracellular reactions; again tyrosine phosphorylation of several proteins of different molecular weight has been observed following fibrinogen binding to GP IIbIIIa and even more following aggregation.[118–120] Since this phosphorylation is inhibited by cytochalasin D, it might well be that this phosphorylation induces cytoskeletal rearrangements.[121] Integrin-initiated tyrosine phosphorylation is dependent on the β subunit cytoplasmic domain.[122]

Weak aggregating agents or low concentrations of stronger ones cause reversible ('primary') aggregation, whereas stronger stimuli cause an irreversible ('secondary') aggregation which is associated with prostaglandin synthesis and the release reaction. It is the aggregation reaction itself which causes TxA_2 synthesis. The substances released from the dense granules, in particular ADP, also play an important role in enhancing secondary aggregation induced by other agonists. Irreversible aggregation is thought to be due to stabilization of the fibrinogen bridges by thrombospondin, which is released from the α granules and has its membrane receptor on GP IV.[123]

Platelet aggregation (and vessel-wall adhesion) as it occurs in vivo is shear stress-dependent. With high shear stresses (more than 60–80 dynes/cm^2), as occurs in stenotic arteries and in the microcirculation, platelet adhesion and aggregation are largely dependent on vWF, which interacts with GP Ib. GP Ib binding leads to opening of transmembrane calcium channels, an intraplatelet rise in calcium levels and conformational changes in GP IIbIIIa, which than can bind vWF, forming bridges between adjacent platelets. In this situation, vWF is both responsible for platelet activation (through GP Ib) and platelet aggregation (forming bridges between GP IIbIIIa molecules of adjacent platelets). At lower shear stresses, as in non-stenotic arteries or veins, the role of the other platelet activators and of fibrinogen and fibronectin as interplatelet bridge is more important; this situation better resembles the classical in vitro aggregometer studies.[124]

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ATTACHMENT II

This Review is part of a thematic series on **New Directions in Thrombosis**, which includes the following articles:

Nitric Oxide Insufficiency, Platelet Activation, and Arterial Thrombosis

Regulation of Vascular Bed–Specific Prothrombotic Potential

Protease-Activated Receptors in the Vasculature, With an Emphasis on Proliferative Responses of Thrombin

Insights Into Mechanisms of Thrombosis From Genetic Models

Molecular Pathogenesis of Antiphospholipid Antibody Syndrome

Joseph Loscalzo, Guest Editor

Nitric Oxide Insufficiency, Platelet Activation, and Arterial Thrombosis

Joseph Loscalzo

Abstract—Nitric oxide (NO) was originally discovered as a vasodilator product of the endothelium. Over the last 15 years, this vascular mediator has been shown to have important antiplatelet actions as well. By activating guanylyl cyclase, inhibiting phosphoinositide 3-kinase, impairing capacitative calcium influx, and inhibiting cyclooxygenase-1, endothelial NO limits platelet activation, adhesion, and aggregation. Platelets are also an important source of NO, and this platelet-derived NO pool limits recruitment of platelets to the platelet-rich thrombus. A deficiency of bioactive NO is associated with arterial thrombosis in animal models, individuals with endothelial dysfunction, and patients with a deficiency of the extracellular antioxidant enzyme glutathione peroxidase-3. This enzyme catalyzes the reduction of hydrogen and lipid peroxides, which limits the availability of these reactive oxygen species to react with and inactivate NO. The complex biochemical reactions that underlie the function and inactivation of NO in the vasculature represent an important set of targets for therapeutic intervention for the prevention and treatment of arterial thrombotic disorders. **(*Circ Res*. 2001;88:756-762.)**

Key Words: endothelium ■ nitroglycerin ■ *S*-nitrosothiols ■ nitric oxide synthase ■ oxidative stress

Nitrovasodilators have been used to alleviate myocardial ischemia for more than a century, and the recognition that they simulate endogenous endothelial nitric oxide (NO) represents one of the most important biological discoveries in cardiovascular biomedicine in the last 20 years.[1] Their antianginal effects have been mostly attributed to relaxation of vascular smooth muscle and resulting arterial vasodilation, which leads to improved perfusion and oxygen delivery. This class of drugs also manifests antithrombotic activity, principally by inhibiting platelet function. First shown by Hampton et al[2] in 1967, the antiplatelet effect of nitroglycerin remained controversial for many years because of the supraphamacological doses required to inhibit platelet aggregation in vitro and the lack of evidence for a direct antiplatelet effect in vivo. Akin to its effects in vascular smooth muscle cells,[3,4] nitroglycerin also inhibits platelet aggregation by activating guanylyl cyclase,[5,6] and this inhibitory action can be potentiated by maintaining intracellular thiol redox state.[6] These observations, coupled with the recognition of the role of platelet-dependent thrombus formation in acute coronary syndromes, led to renewed interest in the antiplatelet effects of nitrovasodilators and, eventually, of NO itself.

In 1985, we reported the case of a 29-year-old woman with a hypertensive crisis treated with sodium nitroprusside for blood pressure control who sustained an intracerebral hemorrhage after being normotensive on therapy for 24 hours.[7] We showed that her bleeding time was prolonged, in vitro platelet aggregation was attenuated in the presence of *N*-acetyl-L-cysteine, and these abnormalities returned to normal with discontinuation of the sodium nitroprusside. These

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data were confirmed in a prospective assessment of platelet function in patients treated with intravenous nitroglycerin; only on restoring platelet thiol stores by addition of *N*-acetyl-L-cysteine ex vivo was the inhibitory action of nitroglycerin apparent.[8] Furthermore, in an animal model of acute platelet-mediated coronary thrombus formation, pharmacologically relevant concentrations of intravenous nitroglycerin can inhibit platelet-dependent cyclic flow reductions, and this effect can be potentiated by *N*-acetyl-L-cysteine.[9] Since these initial observations, several studies have confirmed the antiplatelet effects of nitrovasodilators in vivo and the importance of the vascular redox state and oxidative stress on nitrovasodilator metabolism and action.[10,11]

Endothelium-Derived NO and Inhibition of Platelet Function

In Vitro Studies

The identification of endothelium-derived relaxing factor as NO or a closely related derivative thereof[12,13] and the demonstration that endothelial NO has antiplatelet effects[14,15] raised the question of mechanism. Analogous to assessing the importance of thiols in the action of nitrovasodilators, we first examined the effects of thiols on endothelial NO and demonstrated that in addition to undergoing oxidation to nitrite and nitrate, reacting with superoxide anion to form peroxynitrite, and reacting with heme iron to form the charge-transfer complex required to activate guanylyl cyclase, NO and oxygen or peroxynitrite can react with thiols to form *S*-nitrosothiols.[16,17] These latter compounds serve as stable reservoirs of NO, which can be transferred to and from protein-bound pools, such as *S*-nitroso-serum albumin,[18] by *trans-S*-nitrosation reactions.[19,20] Recent studies also suggest that *S*-nitrosothiols can be stored by platelets and released during heterotypic cellular interactions.[21]

N-Acetyl-L-cysteine potentiates the antiplatelet effect of endothelial NO,[22] and this action can be mimicked by the *S*-nitrosothiol *S*-nitroso-*N*-acetyl-L-cysteine.[23] *S*-nitroso-*N*-acetyl-L-cysteine inhibits both thrombin-induced and U-46619 (a stable thromboxane A_2 analogue)-induced expression of platelet surface P selectin (a granule protein), CD63 (a lysosomal protein), and the calcium-dependent active conformation of the heterodimeric fibrinogen-binding integrin glycoprotein IIb/IIIa (α_{2b}/β_3).[24] This suppression of the conformational change in glycoprotein IIb/IIIa required for fibrinogen binding is associated with suppression of intracellular calcium flux and demonstrable reduction in both the affinity (2.7-fold increase in K_d) and number (50% decrease) of fibrinogen-binding sites on the platelet surface.[23] Inhibition of cytosolic calcium flux with exposure to strong platelet agonists like thrombin or U46619 seems to be a consequence of inhibition of capacitative calcium influx resulting from enhanced sarcoplasmic reticulum/endoplasmic reticulum calcium-ATPase–dependent refilling of calcium stores.[25] *S*-Nitroso-*N*-acetyl-L-cysteine–dependent reduction in fibrinogen binding is dose-dependent and correlates strongly with NO-dependent activation of platelet guanylyl cyclase and cGMP accumulation.[23]

Activation of platelets is associated with activation of another important signaling pathway, the phosphoinositide 3-kinase (PI3-kinase) pathway. PI3-kinase represents a family of ubiquitous enzymes involved in a variety of cell functions, including cytoskeletal rearrangements. These enzymes have the capacity to phosphorylate lipids and proteins. They catalyze the phosphorylation of the inositol ring at the D3 position in a variety of phosphoinositide substrates as well as the phosphorylation of protein serine moieties. The p85/p110 isoform of PI3-kinase was the first to be identified in platelets and is activated by several G protein–linked receptors, including the thrombin receptor protease-activated receptor-1 (PAR-1).[26,27] Activation may involve either a nonreceptor tyrosine-phosphorylated intermediate[28] or may occur directly by some isoforms of G protein subunits.[29,30] Activation of platelets by thrombin results in translocation of PI3-kinase to the cytoskeleton at sites of integrin-dependent focal adhesions, where the enzyme is believed to play an important role in the cytoskeletal reorganization and conformational change in glycoprotein IIb/IIIa required for irreversible fibrinogen binding.[31] Inhibition of this enzyme in platelets by the fungal metabolite wortmannin leads to impaired platelet aggregation and enhanced disaggregation.

Having previously shown that nitrovasodilators can induce platelet disaggregation[32] and platelet PI3-kinase renders platelet aggregation irreversible, we recently examined the effect of the *S*-nitrosothiol *S*-nitroso-glutathione on platelet PI3-kinase.[33] These studies showed that the NO donor inhibits the thrombin receptor–activating peptide stimulation of PI3-kinase activity associated with tyrosine-phosphorylated proteins in immunoprecipitates and of p85/PI3-kinase associated with the src family kinase member lyn. The activation of PI3-kinase complexed with lyn requires the activation of lyn itself and other tyrosine kinases, and inhibition of this process by the NO donor is cGMP-dependent and likely involves inhibition of the dephosphorylation of lyn required for its activation. The effect of PI3-kinase activity on platelet Akt[34,35] and its relationship to platelet NO synthase activity have not yet been reported.

The inhibitory effect of endothelial NO, *S*-nitrosothiols, and nitrovasodilators on platelet activation is analogous to that of prostacyclin and its analogues. However, there are two notable differences between the antiplatelet effects of these two classes of inhibitor: unlike NO, prostacyclin-mediated platelet inhibition is cAMP-dependent; and, also unlike NO, prostacyclin has no effect on platelet adhesion.[36] In contrast to the clear inhibitory effect of NO donors on the expression of the active conformation of platelet glycoprotein IIb/IIIa, NO donors have no effect on the expression of the integrin glycoprotein Ib/IX,[24] suggesting that inhibition of platelet adhesion may be a consequence of inhibiting interactions between von Willebrand factor and glycoprotein IIb/IIIa. We recently synthesized an *S*-nitrosated derivative of the recombinant von Willebrand factor fragment AR545C and found that this molecule effectively and potently inhibited both platelet aggregation and adhesion in vitro and in vivo.[37,38] Recent data also demonstrate that inhibition of platelet adhesion to collagen, in particular, seems to depend on the generation of cGMP[39] and that this effect can be mimicked by the application of an NO-releasing protein (poly-*S*-nitrosated BSA) to the endovasculature.[40] Owing to their different mechanisms of action, platelet inhibition by these two endothelial products is likely synergistic.[32]

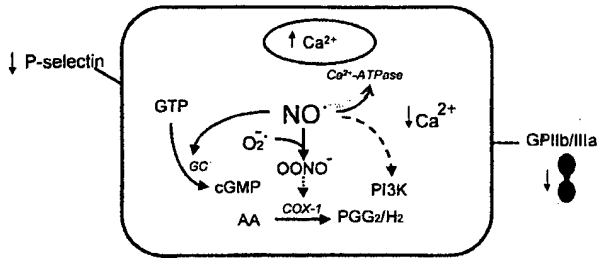

Figure 1. NO effects on platelet signaling and function. NO, derived from endothelial cells or from platelets, suppresses platelet activation by activating guanylyl cyclase (GC), leading to an increase in the conversion of GTP to cGMP, enhancing calcium ATPase–dependent refilling of intracellular calcium stores, and inhibiting the activation of PI3K. As a result of second-order effects mediated by the first two of these signaling systems, intracellular calcium flux (Ca^{2+}) is suppressed, leading to suppression of P-selectin expression and of the active conformation of glycoprotein IIb/IIIa (GPIIb/IIIa) required for binding fibrinogen (trinodular structure). NO also reacts with superoxide to form peroxynitrite ($OONO^-$), which can react with protein tyrosine residues on cyclooxygenase-I to inhibit enzyme conversion of arachidonic acid (AA) to prostaglandins G_2 and H_2 (PGG_2/H_2), with a resulting reduction in thromboxane A_2 synthesis. Solid arrows indicate activation; dashed arrows, inhibition.

The importance of the antiplatelet effects of endothelial NO has been additionally confirmed by studies showing that overexpression of endothelial NO synthase in cultured endothelial cells inhibits platelet aggregation.[41] In addition, NO can inhibit both platelet 12-lipoxygenase and cyclooxygenase-1,[42] in the latter case by reacting with superoxide to form peroxynitrite, which reacts with the enzyme to form a 3-nitro-tyrosine residue.[43]

In Vivo Studies

The studies reviewed thus far show clearly that NO impairs platelet function in vitro by a variety of mechanisms (summarized in Figure 1). These studies were all performed in erythrocyte-free systems. Some investigators have suggested that the affinity of hemoglobin for NO should render these in vitro observations irrelevant in vivo[44]; however, the hydrodynamic effects of the flowing intravascular red-cell mass lead to a partitioning of platelets close to the low-shear boundary of the endothelial surface, which facilitates direct diffusional access of circulating platelets to endothelial NO, limiting kinetic competition of erythrocytic hemoglobin for the free radical. Consistent with this argument, the in vivo relevance of the in vitro observations was first confirmed in a series of experiments in which the effects of another *S*-nitrosothiol, *S*-nitroso-serum albumin, administered intravenously to dogs with an acutely deendothelialized, stenosed coronary artery (Folts model) was studied.[45,46] We had previously demonstrated that *S*-nitroso-serum albumin represents an important in vivo reservoir of NO from which low-molecular-weight *S*-nitrosothiols, such as *S*-nitroso-L-cysteine or *S*-nitroso-glutathione, can be derived by thiol-*S*-nitrosothiol exchange reactions.[19,20] Furthermore, we showed recently that these *trans*-nitrosation reactions can be catalyzed by cell-surface protein-disulfide isomerase to facilitate transfer of NO from an extracellular *S*-nitrosothiol to an

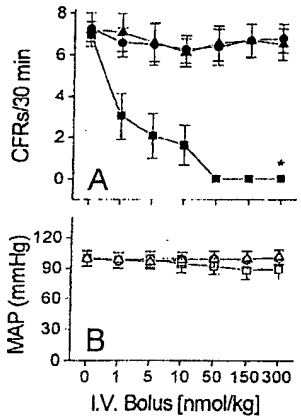

Figure 2. Effect of *S*-nitroso-BSA (SNO-BSA) on cyclic flow reductions (CFRs) in a canine coronary model of periodic platelet deposition. Animals were prepared as described previously.[43] After 40 minutes of continuous CFRs, SNO-BSA (1 to 300 nmol/kg) (squares) was administered as an intravenous bolus over 1 to 2 minutes. Controls included the starting materials for synthesizing SNO-BSA, ie, $NaNO_2$ (triangles) and BSA (circles), prepared in parallel with SNO-BSA. A, Effect of these agents on CFRs (closed symbols). B, Effect of these agents on mean arterial pressure (MAP) (open symbols). Results are mean±SEM, determined from 6 animals. *$P<0.001$ vs $NaNO_2$ or BSA.

intracellular NO acceptor.[47] Using this model, we found that *S*-nitroso-serum albumin dose-dependently inhibited platelet-mediated cyclic flow reductions with an IC_{50} of ≈0.8 nmol/kg (Figure 2A), which translates into an estimated steady-state plasma concentration of ≈5 nmol/L. Importantly, there was only a very modest effect of the NO donor on mean arterial pressure over the range of concentrations used in this study (Figure 2B), suggesting that the antiplatelet effect of *S*-nitroso-serum albumin is more potent than the vasodilator activity in this model of acute coronary syndromes. These animal data were confirmed in patients undergoing coronary angioplasty in whom suppression of platelet activation by the infusion of *S*-nitroso-glutathione occurred at concentrations of the *S*-nitrosothiol that had no effect on blood pressure[48] and in a rat model of thromboembolic pulmonary hypertension in which inhaled NO inhibited platelet aggregation and platelet-mediated pulmonary thrombosis.[49] Furthermore, inhibition of endothelial NO synthase shortened bleeding time in human volunteers,[50] and the coating of artificial surfaces with NO-releasing polymers suppressed platelet adhesion in vivo.[51,52] Thus, ample data in animals and humans support the view that endothelial NO and NO donors have important antiplatelet effects in vivo, especially in the setting of vascular disease.

Platelet-Derived NO and Inhibition of Platelet Recruitment

Under normal conditions of blood flow and shear stress, the vascular source of NO acting on platelets is likely derived from biochemical agonist- and shear-dependent release of endothelial NO.[53] However, under conditions of endothelial dysfunction or denudation, especially in the setting of an acute coronary syndrome, other sources of NO may become important in regulating platelet responses. A constitutive NO

synthase has been found in both human platelets and megakaryocytic cells,[54,55] and this isoform is active.[56] Using an NO-selective microelectrode adapted to a platelet aggregometer, Freedman et al[57] recently showed that this platelet-derived NO not only modestly modulates platelet activation to strong and weak agonists but, more importantly, markedly inhibits platelet recruitment to the growing platelet thrombus. These in vitro findings were confirmed in an animal model of deficient platelet–derived NO, the *Nos3*-null mouse. In this model, Freedman et al[58] found that there is no detectable *Nos3* gene in marrow cells, that their platelets generate no detectable NO on activation, that the bleeding times of *Nos3*-null mice are correspondingly shorter than those of wild-type mice, and that the bleeding times in wild-type mice rendered thrombocytopenic with carboplatin and transfused with platelets from *Nos3*-null mice are shorter than those of mice transfused with platelets from normocytopenic wild-type mice.[58] Ex vivo platelet recruitment experiments using flow cytometry and platelets from *Nos3*-null and wild-type mice confirmed the importance of platelet-derived NO in attenuating platelet recruitment to the growing platelet thrombus.[58] Thus, endothelial- and platelet-derived NO pools both contribute to normal hemostatic function, and a deficiency of either pool enhances hemostatic response to acute vascular injury.

Factors that enhance platelet-derived NO synthesis include α-tocopherol, by inhibiting protein kinase C[59,60]; statins, by increasing expression of NO synthase in platelets (as in endothelial cells)[61,62]; L-arginine, by increasing NO synthesis; and intracellular thiol pools, by enhancing synthesis of *S*-nitrosothiols and limiting oxidative inactivation of NO.[63] By reducing intracellular calcium flux required for the activation of platelet NO synthase, cyclooxygenase inhibitors can reduce platelet-derived NO generation,[64] as can risk factors for atherosclerotic vascular disease[65,66] (vide infra).

The clinical relevance of the importance of platelet-derived NO in patients with acute coronary syndromes was recently examined. Studying 87 consecutive patients undergoing coronary angiography, 37 with stable angina and 50 with unstable angina or an acute myocardial infarction, we found that platelets from patients with acute coronary syndromes produced significantly less NO than did those from patients with stable angina pectoris (0.26 ± 0.05 versus 1.78 ± 0.36 pmol/10^8 platelets, $P=0.0001$).[67] Because platelet activation has been implicated in the formation of thrombus in patients with acute coronary syndromes, we concluded from this study that an impairment of platelet-derived NO production may contribute to the pathophysiology of this class of atherothrombotic syndromes.

NO Insufficiency and Arterial Thrombosis

The potential in vivo relevance of the antiplatelet effects of NO reviewed thus far have been amply demonstrated in the dog model of acute coronary syndromes (Folts model)[45,46] and in patients with acute coronary syndromes.[67] Yet the animal model suffers from the artificiality of its highly platelet-dependent thrombotic response, and the patient study begs the question of causality, because acute coronary syndromes are accompanied by oxidative stress that may itself inactivate NO, rendering it undetectable with the NO-selective electrode used in the study. Thus, these data support

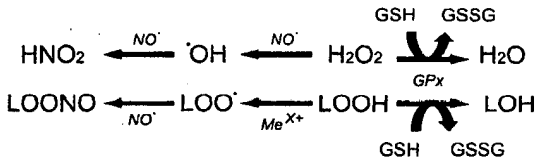

Figure 3. GPx, peroxides, and NO. GPx reduces hydrogen peroxide and lipid peroxides (LOOH) to water and lipid alcohols (LOH), respectively, oxidizing glutathione (GSH) to glutathione disulfide (GSSG) in the process. In the absence of adequate GPx activity, these peroxides can be converted to hydroxyl radical by NO (NO ·)[83] or lipid peroxyl radicals by transition metals (Me^{x+}), which then react with NO to form nitrous acid (HNO_2) or LOONO.

but do not prove the hypothesis that a deficiency of vascular NO promotes arterial thrombosis.

As evidence for the causal relationship between vascular NO insufficiency and arterial thrombosis, we were fortunate to have studied two brothers who presented to our colleague Dr Alan Michelson (University of Massachusetts) with the syndrome of childhood stroke. One boy sustained two separate thrombotic cerebrovascular accidents at ages 13 and 22 months, whereas the other sustained a transient ischemic attack at age 15 months. Routine analysis for known genetic risk factors for arterial thrombosis was negative. In an effort to assess optimal treatment for these children, we evaluated the effect of an NO donor, *S*-nitroso-*N*-acetyl-L-cysteine, on their platelets. In contrast to the platelets of their unaffected sister, mother, and father, as well as age-matched control subjects, the NO donor was completely unable to impair platelet P-selectin expression in response to thrombin and was completely unable to prevent ADP-induced aggregation. Mixing experiments showed that the defect lies in the patients' plasmas; resuspending their platelets in age-matched control plasma led to normal levels of inhibition by *S*-nitroso-*N*-acetyl-L-cysteine. After an extensive search for possible culprit molecular mediators of this inactivation of an NO donor in plasma, results showed that the patients and their mother had a deficiency of the plasma isoform of glutathione peroxidase (GPx-3).[68] This enzyme belongs to a family of selenocysteine-containing proteins, four of which have peroxidase activity. Each of these peroxidases reduces hydrogen peroxide and lipid peroxides to its corresponding alcohol. GPx-3 is the only one of the selenocysteine-containing peroxidases found in the extracellular space and is responsible for most of the hydroperoxide-reducing activity of plasma.[69,70] Previous studies demonstrated that GPx activity potentiates inhibition of platelets by *S*-nitrosothiols and does so both by reducing lipid peroxides to lipid alcohols,[71] thereby preventing the generation of lipid peroxyl radicals that can inactivate NO by forming lipid peroxynitrites[72] (Figure 3), and by catalyzing the liberation of NO from low-molecular-weight *S*-nitrosothiols.[71] The importance of GPx-3 in the regulation of platelet-dependent thrombus formation rests in the fact that activated platelets are rich sources of reactive oxygen species, including superoxide and 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid [12(S)-HpETE][71,73]; and the importance of the enzyme deficiency in the two brothers originally studied was shown by demonstrating the restoration of platelet-inhibitory activity of the NO donor on addition of exogenous (cellular) GPx to their plasmas.[68]

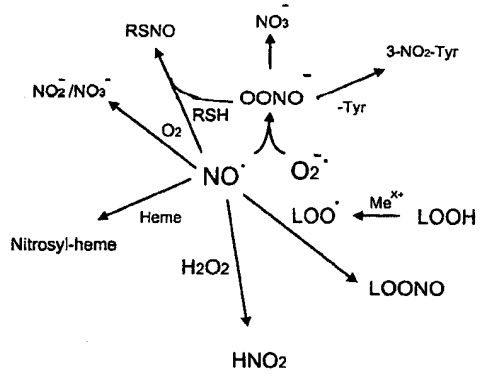

Figure 4. Reactive oxygen species and NO. NO can undergo several fates within platelets, including oxidative inactivation to nitrite (NO_2^-) and nitrate (NO_3^-) and reaction with superoxide anion to form peroxynitrite ($OONO^-$). Peroxynitrite can react with tyrosyl residues in proteins to form 3-nitrotyrosyl residues and with thiols to form *S*-nitrosothiols (RSNO); the latter can also form by the reaction of NO with thiols in the presence of oxygen. By reacting with heme iron, nitrosyl-heme charge-transfer complexes can form, accounting for the biological activation of guanylyl cyclase by NO. Lipid peroxides (LOOH) can yield lipid peroxyl radicals (LOO ·), which can react with NO to form lipid peroxynitrites (LOONO), and hydrogen peroxide can react with NO to form nitrous acid (HNO_2) and hydroxyl radical.[83] Each of these reactions and the reactants that engage in them are present in platelets and in the extracellular microenvironment of activated platelets.

Recent studies expanded this initial observation in a single family to seven families with (familial) childhood stroke. In each of these families, a deficiency of GPx-3 was detected in affected family members, and pedigree analysis suggested that the deficiency is inherited as an autosomal dominant trait. Interestingly, the magnitude of the deficiency correlated with the extent of P-selectin expression measured by flow cytometry in response to thrombin.[74]

In an attempt to identify the basis for the molecular defect, we used single-strand conformational polymorphism (SSCP) analysis of the GPx-3 gene. The gene structure comprises 5 exons, the second of which contains the selenocysteine codon TGA[75]; the 3′-untranslated region contains an element of putative secondary structure (a stem loop) that is required for recognition of TGA as a selenocysteine codon rather than as a stop codon.[76] We performed SSCP using primers for the five exons, the putative promoter, and the 3′-untranslated region of the GPx-3 gene on DNA from 100 individuals with stroke younger than 35 years of age to identify potential mutations or polymorphisms.[77] We sequenced those regions that showed differences by SSCP analysis and identified a novel polymorphism in the promoter region that confers an independent risk for stroke in these individuals (relative risk=2.5; confidence interval, 1.31 to 4.81). The possible role of this polymorphism in the regulation of GPx-3 gene expression has yet to be characterized.

The importance of vascular NO in preventing thromboembolic strokes is additionally supported by recent animal data showing that inhibition of NO synthase leads to platelet accumulation in the cerebral vasculature.[78] The importance of (cellular) GPx (ie, GPx-1) activity in endothelial antiplatelet actions has been shown by the dependence of an aspirin-sensitive inhibitory activity (prostacyclin synthesis and stability) on selenide content of the endothelial cell.[79]

Reactive Oxygen Species, Inactivation of NO, and Arterial Thrombosis

NO is a reactive free radical that can participate in several types of redox reactions, some that mediate its biological effects and others that limit its activity (Figure 4). Examples of the first type of reaction include the reaction of NO with heme iron (ie, nitrosylation), which is responsible for the activation of guanylyl cyclase, and the reaction of NO with thiol groups in the presence of oxygen (or via peroxynitrite intermediates) to form *S*-nitrosothiols, which stabilize bioactivity. Inactivation of NO occurs largely through oxidative reactions mediated by reactive oxygen intermediates, including superoxide, hydrogen peroxide, and lipid peroxyl radicals generated from lipid peroxides[17] as well as the F_2-isoprostane 8-epi-prostaglandin F_2.[80] These reactive oxygen intermediates are found in a variety of vascular disorders, including hypertension, hypercholesterolemia, diabetes mellitus, atherothrombosis, and the endothelial dysfunction that underlies or accompanies these disorders.[81]

Platelets are themselves a rich source of reactive oxygen species, including superoxide and 12(S)-HpETE.[71,73] The consumption of oxygen by activated platelets is robust and serves as a mechanism to enhance the aggregation response through the formation of proaggregatory prostanoid derivatives of arachidonic acid. This oxygen-dependent autoamplification of the activation of platelets can, in the theoretical extreme, proceed without restraint. For this reason, antioxidant mechanisms have evolved to limit unbridled expansion of the platelet aggregate. Endothelium- and platelet-derived NO represent one class of regulatory molecule that impairs platelet activation and recruitment to the growing thrombus. In addition, the antioxidant enzyme GPx-3 represents another regulatory molecule that, in conjunction with extracellular superoxide dismutase found on the endothelial surface, serves to inactivate those reactive oxygen intermediates that can inactivate NO either directly or via other radical derivatives. The formation of peroxynitrite by the reaction of either endothelial- or platelet-derived NO with either endothelial- or platelet-derived superoxide represents yet another mechanism for limiting the reactive oxygen species–dependent thrombotic response, both by limiting available superoxide and by impairing thromboxane A_2 generation via the 3-nitrotyrosine–dependent inactivation of cyclooxygenase-1[41] and by *S*-nitrosothiol–dependent inhibition of thromboxane A_2 synthesis.[82] Platelet-dependent arterial thrombotic responses, then, are both a cause and a consequence of excessive oxidant stress in the vasculature, and arterial thrombotic disorders are their clinical counterpart. NO, derived both from the endothelial cell and the platelet, modulates platelet activation, adhesion, and aggregate formation, thereby serving as an important deterrent to platelet-mediated arterial thrombosis. The studies reviewed here clearly show that vascular oxidant stress produced by an excess of oxidants or an acquired or genetically determined deficiency of antioxidant enzymes is a risk factor for arterial thrombosis. Efforts to restore the normal vascular redox balance may provide one therapeutic

avenue for reducing platelet-dependent arterial thrombosis in these individuals.

Acknowledgments

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ATTACHMENT III

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FLAVONOIDS AS SCAVENGERS OF NITRIC OXIDE RADICAL

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Flavonoids are a group of naturally occurring compounds used ,e.g., in the treatment of vascular endothelial damage. They are known to be excellent scavengers of oxygen free radicals. Since the nitric oxide radical ('NO) probably plays a role in this pathology, the 'NO scavenging capacity of the flavonoids was determined. It was found that the flavonoids are very potent 'NO scavengers. The anthocyanidins were found to be more effective scavengers than the hydroxyethylrutosides, which correlated with their therapeutic activity. The values of their scavenging rate constants are only 30 times less active than the very potent endogenous 'NO scavenger haemoglobin. It is speculated that 'NO scavenging plays a role in the therapeutic effect of the flavonoids.

Flavonoids are a group of naturally occurring antioxidants, usually found in plants, fruits and vegetables. As pure compounds or as mixtures, they are clinically used in diseases of the vascular wall involving inflammation and endothelial damage[1-4]. Venoruton, a semi-synthetic hydroxyethylrutoside mixture is used to reduce capillary permeability in chronic venous insufficiency and to protect the endothelial layer of blood vessels in diabetes mellitus[4].

In inflammation and endothelial damage free radicals play a major role. Of most radicals, both beneficial as well as undesirable effects are described [5]. This holds, especially, true for 'NO. On the one hand 'NO is a key mediator in various physiological processes.

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On the other hand 'NO is toxic e.g. as precursor of peroxynitrite [6].

Part of the therapeutic effect of flavonoids has been ascribed to their free radical scavenging capacity. Because of the important role of 'NO, the scavenging of 'NO by therapeutically used flavonoids is studied.

Materials and Methods

Chemicals

Rutin was obtained from Merck and pelargonidin chloride was purchased from Fluka. Cyanidin chloride was purchased from Roth. The hydroxyethylrutosides and trihydroxyethyl quercetin were a generous gift from Zyma (Nyon, Switzerland). NO gas was from Messner Griesman, 99.95% pure.

NO scavenging

Deoxygenated water was purged with 'NO gas for about 1 min. Four µl of the 'NO saturated water was added to 20 ml 50 mM phosphate buffer (pH 7.4) in a thermostatted waterjacketed bath (37°C). The buffer was saturated with N_2 gas and during measurement the test vessel was kept under an N_2 atmosphere. The 'NO concentration was monitored with an iso-NO meter (World Precision Instruments Inc., Sarasota FL, USA) which was coupled to a MacLab interface (ML020 MacLab/8, ADInstruments Ltd, London, England) and an Apple Macintosh computer with 'Chart' software. The decrease in 'NO concentration was followed in time in the presence or absence of flavonoid. The flavonoids, in a stock concentration of 10 mM, were dissolved either in millipore water (tetraHER) or in 100 % DMSO (all other flavonoids). The final DMSO concentration was 1%, which has no effect on the measurements. The final concentration of flavonoid was 100 µM, or lower.

The log K values were calculated by plotting the ln 'NO concentration versus time, which resulted in a straight line. The reaction shows pseudo first order kinetics, as the scavenger is in excess. By dividing the pseudo first order rate constant (ks) by the concentration of scavenger, the scavenging rate constant (K) is obtained:

$$v = -\frac{d[NO]}{dt} = K[S]['NO]$$

$$ks = K[S]$$

Corrections were made for the spontaneous degradation of 'NO (first order rate constant $1.0 \pm 0.1 \times 10^{-3}\ M^{-1}\ s^{-1}$).

Results and Discussion

It is known that some flavonoids are excellent scavengers of free radicals like the hydroxyl radical and superoxide anion radicals [7-9]. In the present study, we found that flavonoids are also excellent 'NO scavengers (table 1). Their activity exceeds that of glutathione 10 to 1000 times. For comparison, we determined the log K of haemoglobin, which was found to be 4.25. This means a K value of $1.78 \times 10^4\ M^{-1}\ s^{-1}$, which is

Table 1. log K values of the flavonoids tested

Flavonoid	log K ± SD ($M^{-1}s^{-1}$)	number of exp.
cyanidin	2.54 ± 0.24	3
pelargonidin	2.60 ± 0.08	3
monoHER	0.95 ± 0.05	4
diHER	0.87 ± 0.05	4
triHER	1.04 ± 0.03	4
tetraHER	0.99 ± 0.03	4
rutin	0.96 ± 0.06	4
haemoglobin	4.25 ± 0.23	2

comparable to the value of 2.1 x 10^4 M^{-1} s^{-1} reported by Alayash et al.[10]. The anthocyanidins, cyanidin and pelargonidin, appear to be more potent scavengers of NO compared to the hydroxyethyl rutosides. The halflife of 'NO is decreased by 50 % by adding only 5μM of pelargonidin, the most potent flavonoid studied (Fig. 1). The 'NO scavenging potency correlates with the therapeutic efficacy since the anthocyanidins are known to be more effective than the hydroxyethylrutosides in reducing capillary permeability and fragility and in their anti-inflammatory and anti-oedemic activities [11].

As mentioned above, 'NO has both beneficial as well as undesirable effects. 'NO is a mediator of inflammation [12, 13] and plays an essential role in the defence mechanism of macrophages against micro-organisms [14]. 'NO generated by inflammatory cells is toxic, probably after reaction with superoxide anion, which gives rise to peroxynitrite. Once protonated, peroxynitrite decays rapidly to form the reactive HO· and the stable NO_2· radical, as suggested by Beckman et al. [15]. Scavenging of these 'NO radicals will contribute to the therapeutic effect of the flavonoids.

In blood vessels 'NO is produced by endothelial cells, e.g. upon stimulation of muscarinergic receptors. The 'NO diffuses to the vascular smooth muscle cells where 'NO-induced cGMP formation leads to muscle relaxation. Scavenging of this 'NO will lead to vasoconstriction and, possibly, to vascular damage.

Flavonoids appear to accumulate between the endothelial layer and the vascular smooth muscle cells, where a high local concentration is reached [16]. This would favour the scavenging of the endothelial derived NO that is responsible for vasodilatation. However,

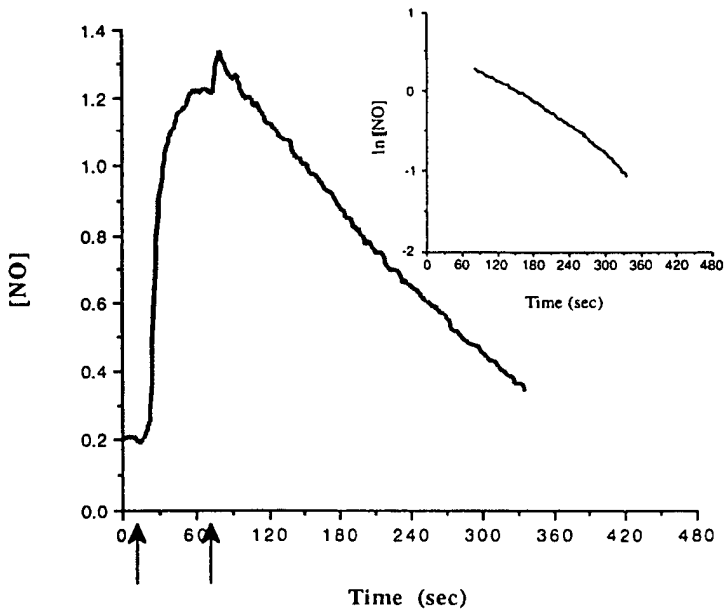

Fig. 1. A representative experiment with 5 µM Pelargonidine. At the timepoint of the first arrow 4 µl of NO saturated water is added to the testvessel. At the timepoint of the second arrow 200 µl of a 0.5 mM solution of Pelargonidine is added. The inset represents the ln [NO] vs time curve.

at the site where the flavonoids accumulate also arteriosclerosis begins in a process where undoubtedly 'NO radicals display an unfavourable role. A correlation has been found between the intake of flavonoids and a low incidence of cardiovascular diseases (the Zutphen Elderly study [17]). Therefore, it is tempting to speculate that 'NO scavenging of flavonoids plays an important role in their therapeutic effect.

If indeed 'NO scavenging is one of the major protective features of the flavonoids, we wonder how the scavenger is able to discriminate between the wanted and the deleterious 'NO. Probably the local distribution of the flavonoid has to be taken into consideration.

In conclusion, we demonstrate the potent 'NO scavenging effect of some flavonoids. It is speculated that this feature of the flavonoids is of importance in the therapeutic effect of these naturally occurring antioxidants.

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ATTACHMENT IV

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MINI REVIEW

PLATELETS AND ANTIPLATELET DRUGS

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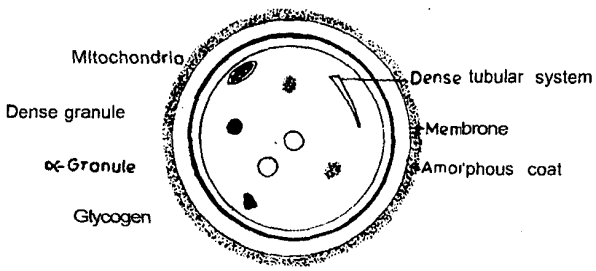
Platelets serve a variety of functions in the circulation. Their major purpose is participation in haemostatic mechanisms. Platelets, approximately 2 μm in diameter and 200,000-400,000/cu mm, are produced by the megakaryocytes in the bone marrow' and have a life span of 10 days. They have no nucleus or DNA, but are capable of synthesising fatty acids, phospholipids etc. A small amount of RNA is present and protein synthesis has been demonstrated.[2] The cell membrane is covered with an amorphous coat and its exact function is unknown. The cell membrane contains glycoproteins (GPIa, GPI_b, $GPII_b$, $GPIII_a$) which acts as receptors for various ligands and play an important role in the platelet aggregation. Next to the membrane is the microtubule, a circumferential band of hollow cylindrical structure (Figure 1). Platelets have an extensive, intricate canalicular system in contact with the extracellular fluid which facilitates secretion of granules. There are three types of granules identified in the cell. First type of granules are alpha-granules, which contain a variety of coagulation factors like beta-thromboglobin (BTG), fibronectin, fibrinogen, FVIII-vWF, thrombospondin, platelet factor-4 (PF_4), and platelet-derived growth factor (PDGF). A second type of granules are, dense granules which store ADP, serotonin and calcium. The third type called lysosomal granules, contains hydrolases and related enzymes. Platelets contain actin and myosin.

Figure 1. Diagrammatic representiation of electron micrograph of platelet



Normally in the circulation the platelets are nonadherent to each other and to vascular endotheiium. This unique property is disturbed when they come in contact with subendothelial surface during vessel disruption.

In this review the physiology of platelet function and receptors, the disorders of their function and the probable mechanism of action of antiplatelet drugs will be discussed.

1. **Physiology of platelet function:** Platelet aggregation is necessary for endothelial repair and thrombogenesis. Aggregation consists of the following sequence of events.

a. Endothelial injury: Vascular injury produces endothelial denudation and exposes subendothelial connective tissue which activates the platelets.[3] Mechanical injury such as surgery, vascular instrumentation by needles or catheters and acute hypertension can cause damage to endothelial cells.[4] Antibodies, circulating immune complexes, endotoxin, viral infections etc. can induce direct endothelial cytotoxicity.[5-7] Chemical endothelial injury has been attributed to hyperlipidemia, bile salts, radiologic contrast dyes and some chemotherapeutic agents.' Endothelial damage is also seen in anoxia, smoking, diabetes mellitus, coronary atherogenesis etc.[5,9]

b. Platelet activation: Vascular injury denudes endothelium and exposes subendothelial structures to circulating blood (Figure 2). Platelets are activated by contact with the subendothelial collagen, assume spherical shape from discoid and adhere to collagen by putting out pseudopodia. During adhesion, interaction of GPI_a with subendothelial collagen and GPI_b

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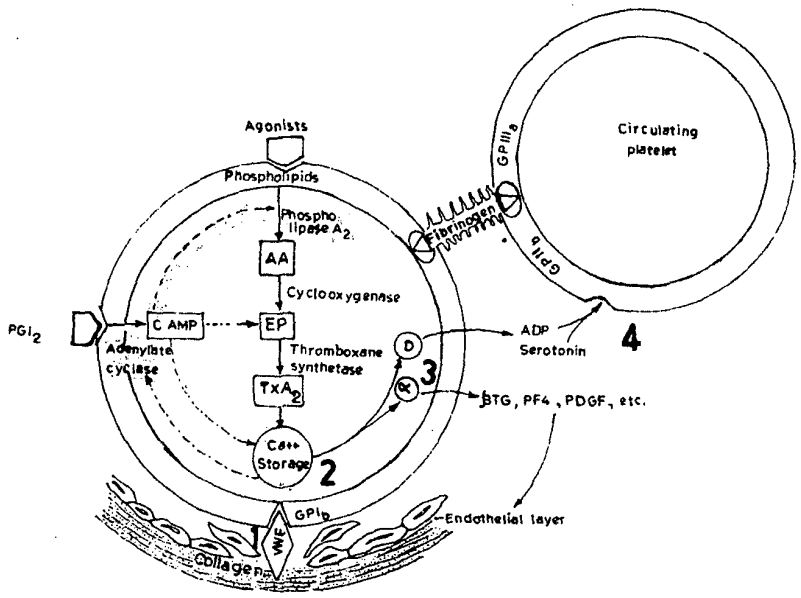
Figure Diagrammatic representation of activation of platelet.
1. Adhesion, 2. Contraction and shape change, 3. Release reaction, 4. Secondary aggregation
GP = Glycoprotein, CAMP = Cyclic adenosine phosphate, AA= Arachidonic acid, EP = Endoperoxide, TXA_2 = Thromboxane A_2, D = Dense granule, α = Alpha granule, PF_4 = Platelet factor 4
——→ Stimulation, ····· _ ——— ⇢ Inhibition



with plasma von Willebrand factor (vWF) takes place. 10 Endothelial cells and megakaryocytes synthesise vWF.[11,12]

Platelet activation results in the elevation of free cytoplasmic calcium (Ca^{2+}) which binds to the intracellular calcium regulatory protein, calmodulin to form the Ca^{2+}-calmodulin complex which helps to activate the myosin kinase system. Active myosin kinase facilitates phosphorylation of myosin and thereafter actin-myosin interaction and granule secretion. This secretion is also known as "Release reaction". It involves release of dense granule, alpha-granule constituents and activation of platelet membrane phospholipase complex to generate thromboxane A2 (TXA_2).[13] Both ADP and TXA2 act synergistically to recruit more circulating platelets causing them to change shape and attach both to each other and to platelets already adherent to the damaged vessel wall. Exogenous ADP (derived from red cells or extravascular tissue) may directly initiate platelet aggregation. Epinephrine, serotonin and thrombin also induce aggregation directly. All these direct actions are mediated through specific binding sites (Table 1). The term 'primary aggregation' refers to this direct aggregation. Aggregation mediated through the release reaction is known as 'secondary aggregation'.

2. Platelet receptors: Platelet cell membrane possesses a number of receptors in addition to glycoprotein receptors (Table 1).

a. Serotonergic receptors: The $5HT_2$ or S_2 receptors are present in platelets and are similar to those on vascular smooth muscle. Serotonin is taken up from the blood and is stored by the platelets so that very little is present in the plasma.[14-16] It is released from platelets during vascular injury, cardiopulmonary bypass etc. Serotonin released by positive feed back induces further aggregation activating S_2-receptors.[17,18]

b. Adrenoceptors: In certain conditions of stress, physical exercise, norepinephrine infusion and smoking, catecholamine levels are increased and may precipitate thrombosis activating the haemostatic mechanisms.[19,20] Characterisation of alpha-adrenoceptors shows a significant population of α_2-subtype receptors.[21,22] Epinephrine elicites the aggregation directly through α_2-adrenoceptors. With prazosin as the binding agent α_1- adrenoceptors were not detected in patelets.[23] On the basis of

Table 1. Platelet receptors and their ligands

Receptors	Ligand
GPI_a	Collagen
GPI_b	vWF
$GPII_b$-III_a Complex	Fibrinogen
α_2	Epinephrine, Norepinephrine
β_2	Epinephrine
S_2	Serotonin
V_1	Vasopressin
IPR	Imidazolines[51-53]

' Thrombin, PAF, TXA_2, Adenosine

' Binding sites identified
GP=Glycoprotein, S=Seroninin, V=Vasopressin, PR= Imidazolines preferring receptors.

Table 2. Disorders of platelet function

Disorder	Reason
Von Willebrand's disease	Decreased levels of vWF
Bernard-Soulier syndrome	Absence of GPIb
Glanzmann's disease	Absence of GPIIb-IIIa complex
Chediak-Higashis syndrome	Granule storage defect

differing responses to oxymetazoline, Bylund and coworkers designated the platelet adrenoceptors as α_2-A subtype.[24] Human platelets possess, in addition to α_2-adrenoreceptors, β_2- adrenoreceptors that mediate platelet inhibition by stimulation of adenylate cylase. The outcome of action by epinephrine on platelets, depends on the relative population of α_2 and β_2 adrenoceptors which vary among species.[25,26]

Other platelet receptors taking part in aggregation have been given in the Table 1.[27]

3. Disorders of platelet function: Decreased platelet function due to abnormalities of platelet cell components are given in the Table 2.[28]

4. Antiplatelet agents: They can be considered under various categories according to their echanism of interference in the pathway of platelet activation (Table 3).

Table 3. Antiplatelet agents

a. **Drugs inhibiting membrane phospholipase activity:**
Mepacrine, corticosteroids, clofibrate

b. **Drugs Inhibiting arachidonic acid metabolism**
Cyclooxigenase inhibitors:
Aspirin, sulphinpyrazone

Thromboxane synthetase inhibitors:
Dazoxiben, imidazoles

c. **Drugs that increase c-AMP**
Adenylate cyclase stimulation:
Prostacyclin

Phosphodiesterase inhibition
Dipyridamole, enprofylline

d. **Drugs acting on platelet membrane**
Ticlopidine

e. **Drugs acting as receptor antagonists**
thromboxane A2 antagonists:
Daltroban

$5HT_2$ (S_2) antagonists:
Ketanserin

f. **Drugs acting through intracellular calcium**
Diltiazem

g. **Miscellaneous**
Propranolol, organic nitrates, pyridoxine

a. Drugs inhibiting membrane phospholipase activity: Mepacrine, high doses of corticosteroids and clofibrate prevent membrane phospholipase activity and liberation of arachidonic acid from membrane-phospholipids and block the TXA2 synthesis.[29]

b. Drugs inhibiting arachidonic acid metabolism: This group includes platelet cyclooxygenase inhibitors and thromboxane synthetase inhibitors. This type of blockade will not affect adherence of platelets to collagen, collagen-induced release of granular contents and thrombin mediated aggregation and release.[29,30] Dense granule ADP contributes to aggregation in the presence of these inhibitors.[31] Furthermore, these agents do not prevent the release of platelet -derived growth factor (PDGF) from alpha-granule. Smooth muscle proliferation by PDGF in response to vessel damage will continue.[32] Thrombin-mediated platelet aggregation and release occur through arachidonic acid independent pathways.[33] Hence, these drugs do not inhibit thrombin mediated aggregation.

Low doses of aspirin (40 mg/day), inhibit the synthesis of platelet **TXA2 permanently,**[34] while the inhibitory effect on endothelial prostacyline synthesis is reversible.[35]

Sulphinpyrazone is a competitive and reversible inhibitor of platelet cyclooxygenase[29,30] and normalises shortened platelet survival, whereas aspirin does not.[36]

Thromboxane synthetase inhibitors such as dazoxiben, picotamide and ridogrel block the conversion of cyclic endoperoxides to TXA_2.[37,38] These drugs are interesting since they selectively allow endothelial cells to synthesise prostacyclin (PGI_2).[39] However thromboxane synthetase inhibitors have not been adequately tested clinically.

c. Drugs that increase CAMP levels: By binding to membrane receptors, prostaglandins (PGI2, PGE_1, PGD2) elevate platelet cAMP which leads to inactivation of platelet myosin kinase which in turn reduces actin-myosin interaction, platelet contraction and granule secretion.[40,41]

Enprofylline and dipyridamole act by inhibiting platelet phosphodiesterase thereby raising cAMP levels[37,8,42,2] and this prevents platelet adhesion to subendothelial collagen, platelet contraction and release. Similar to sulphinpyrazone, dipyridamole normalises shortened platelet survival and does not prolong the bleeding time.[29,30]

d. Drugs acting on platelet membrane: Ticlopidine has broad spectrum antiaggregatory property. It is a potent platelet function inhibitor and in *vitro* it prevents primary aggregation induced by agonists. *In vivo,* it prolongs the bleeding time and normalises shortened platelet survival.[29] Its mechanism of action may be related to inhibition of ADP-induced exposure of fibrinogen binding sites of glycoproteins (GPIIb-GPIIIa complex).[43]

. Drugs acting through receptors: Though imidazoline group of drugs like clonidine are initially considered to be acting through alpha-adrenoceptors on platelets, more recent evidence shows that they act through nonadrenergic sites called imidazoline preferring receptors (IPR).[44-46] Daltroban blocks TXA2 receptors while picotamide and ridogrel block TXA2 receptors in addition to their inhibitory effect on thromboxane synthetase.[38] Ketanserin, a selective S_2-receptor antagonist inhibits platelet aggregation and release reaction induced by serotonin. After discontinuation, a transient hyperactivity of platelets to serotonin has been observed.[17,47,48]

f. Drugs acting through intracellular Ca^{2+}: Calcium channel blockers like verapamil, diltiazem and nifedipine inhibit platelet aggregation by decreasing the release of ADP, TXA_2 synthesis through blocking intracellular Ca^{2+} release.[49] Diltiazem appears to have the greatest overall antiplatelet activity among the calcium antagnist studied.[50]

g. Drugs acting through less well defined mechanisms: Organic nitrates and beta-adrenergic blocking drugs possess platelet inhibitory effects which are of particular interest in coronary artery disease. Nitroglycerine has been shown to inhibit platelet aggregation induced by a number of agents.[51] The possible mechanism may be through its potentiation of PGI_2 mediated inhibition.[5] It was reported that both racemic and d- propranolol inhibit ADP induced platelet aggregation *in vitro;* dl-propranolo, d-propranolol and physostigmine exhibited significant anti-aggregatory effects, which correlate well with the inhibition of cholinesterase enzyme. Several studies have shown that a cholinergic mechanism may be involved in propranolol effects.[53] It was also shown that propranolol inhibits the generation and release of prostaglandins by platelets.[54] Propranolol diminishes platelet sensitivity to ADP-induced aggregation and this is independent of its beta-adrenoceptor blocking effect.[55] It does not normalise shortened platelet survival time.[36] Abrupt withdrawl of propranolol has been shown to produce rebound platelet hyperaggregability, decreased exercise tolerance and more frequent angina.[56] Pyridoxine acts as an antiplatelet agent probably by binding to fibrinogen and platelet surface.[57]

It may be interesting to note that the morning increase in platelet aggregability is related to changes in posture and is associated with the release of catecholamines. This might bear a relationship to the observed increase in the incident of myocardial infarction early in the morning.[58]

Though most of the molecular mechanisms underlying the activation of platelets are identified and are manipulated by several pharmacological agents, the present knowledge of antiplatelet drugs is hardly satisfactory. A lot remains to be explored and many new target areas for drug action should be identified, which will facilitate the development of more drugs to regulate platelet action. The present trend of research in this field makes one feel optimistic and in the near future the desire for an ideal antiplatelet agents may be fulfilled.

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